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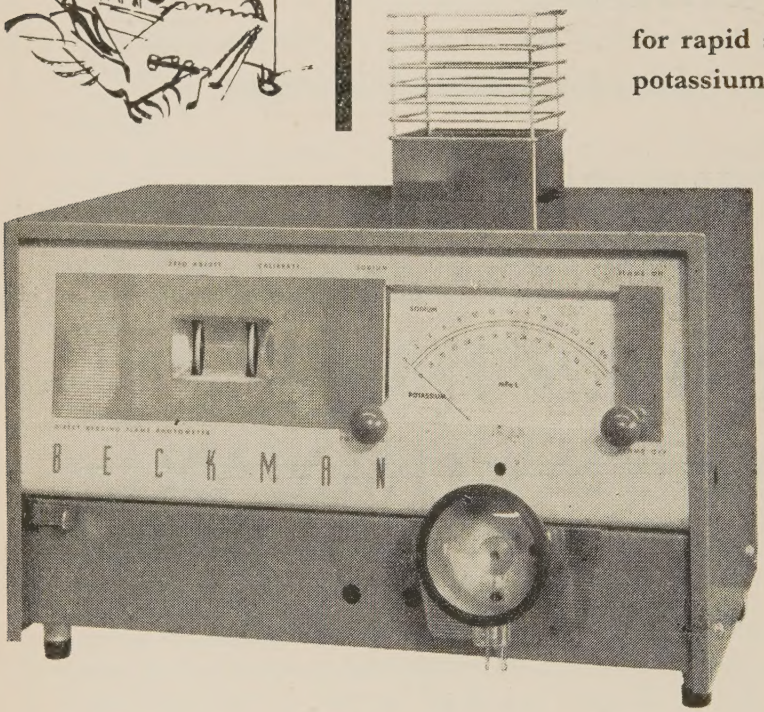

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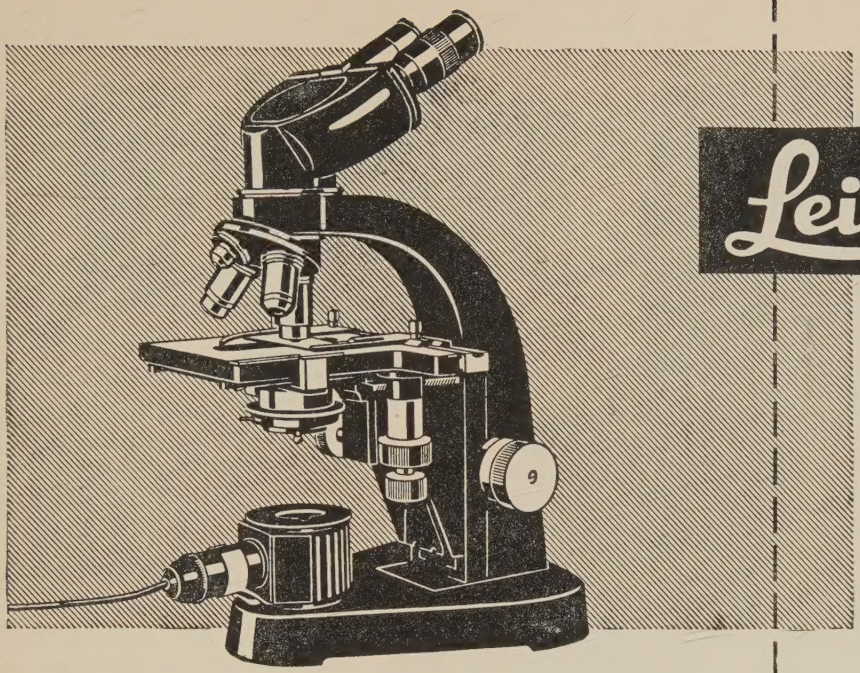

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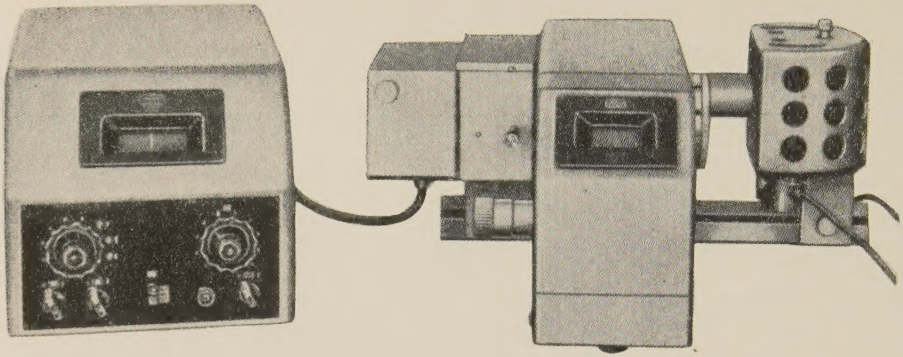

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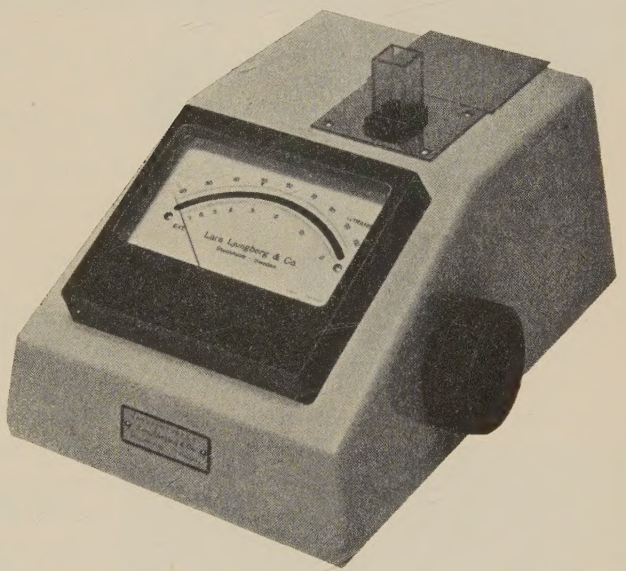

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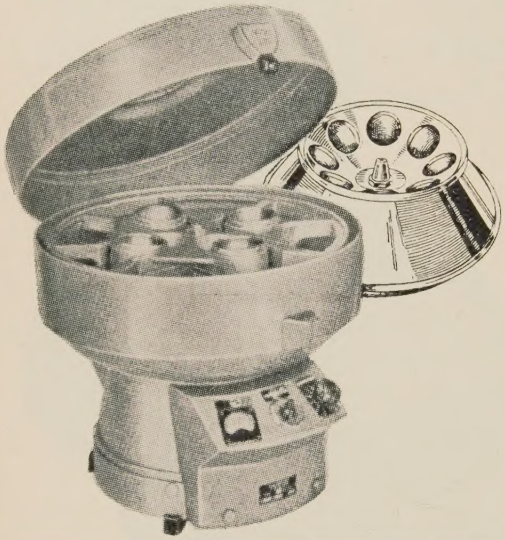

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QUANTITATIVE DETERMINATION OF GLUCOSAMINE

By B. ROSENLUND*

From The Central Laboratory, Rikshospitalet, Oslo

(Received for publication April 10, 1955)

Glycoprotein determinations in serum are the object of increasing interest since it has been found that many diseases, especially those of chronic nature, are accompanied by an increased concentration of the protein-bound polysaccharides in blood. In practice, the carbohydrate fraction is usually determined quantitatively, affording an indirect expression of the glycoprotein concentration. There are several types of carbohydrates in the glycoproteins, the quantitatively most important are mannose, galactose and glucosamine.

Pauly & Ludwig (1922) showed that glucosamine, after acetylation in an alkaline milieu with ethyl acetoacetate or acetyl acetone, yields a pyrrol derivative which, with Ehrlich's reagent in a muriatic alcoholic milieu, gives a stabile, red-violet color. Zuckerkandl & Messiner-Klebermass (1931) employed this principle in their quantitative method for determining glucosamine. Elson & Morgan (1933) improved the method further and introduced acetyl acetone as a standard acetylation agent.

The principle of this method has been adopted in several subsequent modifications, among others by Nilsson (1936), Palmer, Smyth & Meyer (1937), West & Clarke (1938), Blix (1948), Stary, Bursa, Kaleoglu & Bilen (1950) and Südhof & Petrovic (1954). Blix' modification is quantitative within a broad analytical range and gives satisfactorily reproducible results. The neutralization which is one step in the method is carried out after a trial titration in an aliquot, and must often be corrected. This protracts and complicates the analysis. Attempts have been made to titrate directly with methyl red as indicator, as reported by Sundblad and cited by Blix (1948).

A prerequisite for employing this technique, however, is that the indicator does not interfere with the colored product which

* The investigation was carried out with grants from the Norwegian Research Council for Science and the Humanities and from A/S Freia's Medical Fund.

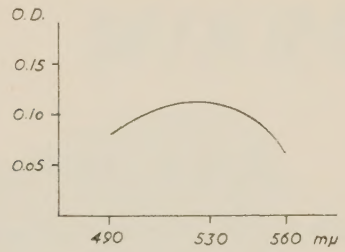

Fig. 1. Absorption curve for test solution without glucosamine, with one drop of methyl red, read against water.

develops in the analysis. A control investigation revealed that this prerequisite is *not* fulfilled, as methyl red has considerable self-absorption at the wave length employed, 530 mμ (Fig. 1, Table I).

It seemed logical to test other indicators, and parallel experiments, with and without bromthymol blue, showed that this indicator does not influence the extinction values at 530 mμ (Table II). As a consequence, direct titration with bromthymol blue as indicator has been introduced in the method.

Furthermore, some investigations have been made regarding certain aspects of the method which seem to be significant for its accuracy and specificity, and some small modifications have been found practical:

NaOH

An accurate turning point is more readily obtained by using 2N NaOH instead of 4N during the titration.

Acetyl acetone.

The reliability of the method is extremely dependent on the purity of this reagent. Acetyl acetone of inferior quality yields a colored product in the blanks with maximum absorption around 530 mμ and will thus influence the analytical result. Palmer *et al.* (1937) have made the same observation but are not able to explain the cause.

Table I. *Parallel analyses without (Blix) and with (Sundblad) methyl red.*

	Blix' modif. Optical density	Sundblad's modif. Optical density	Difference
Glucosamine HCL-standard	0.252	0.380	0.128
Serum	0.408	0.514	0.106

Vacuum distillation at + 70° C of this type of acetyl acetone gave a clear distillate which did not yield any trace of color in the blank (controlled against distilled water) after standing for 24 hours. The residue after distillation was yellow in color, and a blank carried out with this yielded an almost instantaneous strong red-violet color with high extinction at 530 mμ. It may thus be considered as proven that impurities in the acetyl acetone may form condensation products with Ehrlich's reagent and interfere with the analytical results. If the acetyl acetone is yellow or acid, this will usually indicate that it is impure.

Acetylation time and temperature

It is important to standardize the acetylation time, and 1 hour has been employed, whereby the large majority of pathologically increased serum glycoprotein values will give extinction values which follow Lambert-Beer's law.

As to temperature, experiments have shown that variations during the acetylation between 95 and 100° C (in the water bath) do not influence the results (Table III).

Table II. *Parallel analyses with and without bromthymol blue.*

Blank	Analysis	Optical density
With bromthymol blue	Glucosamine standard with bromthymol blue	0.289
Without —»—	—»—	0.289
With —»—	Serum with bromthymol blue	0.344
Without —»—	Serum without bromthymol blue	0.346

Recording of the analytical results

The analyses exhibit increasing extinction values up to 45 minutes after the addition of Ehrlich's reagent. From 45 to 75 minutes after the addition of the reagent the extinction values are constant and photometric readings should be made during this period.

The influence of other factors on the analytical results.

Immers & Vasseur (1952) have pointed out that glucose together with certain amino acids under special conditions may have a considerable influence on the analytical results. Experiments with glucose — lysine HCl — glucosamine HCl mixture, carried out according to the method described below, show that lysine-glucose has no influence of significance on the analytical results even in concentrations far higher than would be expected in biological material (Table IV).

The same authors also find that the absorption maximum for pure glucosamine-HCl solutions decreases somewhat on standing, and at the same time shifts from 530 to 510 mμ. Experiments show that the absorption maximum decreases some, but that it does not shift, even after several days, with the method here described (Fig. 2).

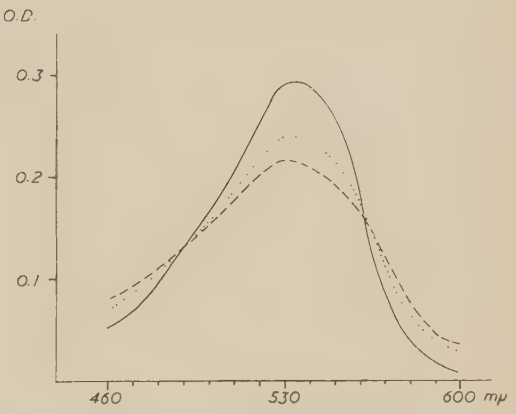

Fig. 2. Absorption curves for a serum glucosamine analysis, 45 min., 3 and 6 days after the addition of Ehrlich's reagent.
– – ——— after 45 minutes
..... after 3 days.
- - - - - - - - - - after 6 days.

METHOD

Reagents

2 N HCl.

2 N NaOH.

Bromthymol blue, 0.1 per cent in alcoholic solution.

Acetyl acetone reagent: 1 ml pure acetyl acetone is dissolved in 25 ml 1.25 N Na_2CO_3. The solution must be freshly prepared.

Ehrlich's ragent: 1.6 g pure para-dimethylaminobenzaldehyde is dissolved in 30 ml concentrated HCl and 30 ml 96 per cent ethanol. This solution may be stored for several days at + 2—4° C.

Procedure

0.1 ml serum is added to 2 ml 2 N HCl and hydrolysed for 12—14 hours in a boiling water bath in a sealed Pyrex test tube with a 5 ml measuring mark. After cooling this is carefully titrated with 2 N NaOH, one drop of bromthymol blue as indi-

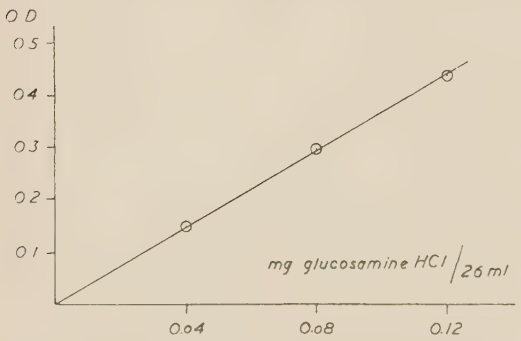

Fig. 3. Standard curve from the author's modification of Elson & Morgan's method.

cator. Distilled water is added to 5 ml. Filter. A 2 ml aliquot of the filtrate is transferred to a 50 ml Erlenmeyer flask with glass cork, and 2 ml acetyl acetone reagent are added. The flasks are placed in a shallow water bath at 95—100° C, and the corks are put in firmly after a few seconds. Acetylation for one hour. Cool and add 20 ml 96 per cent ethanol and 2 ml Ehrlich's reagent while shaking to get rid of CO_2. Read in spectrophotometer after at least 45 minutes at 530 mμ, 10 mm cuvettes. The blank is prepared in the same manner without hydrolysis, with 2 N HCl instead of the hydrolysate. The pale yellow color which develops after hydrolysis of the serum gives a negligible extinction at 530 mμ.

Standard curve

From a standard solution of pure glucosamine HCl containing 100 mg/100 ml, 0.1, 0.2 and 0.3 ml were removed, 2 ml 2 N HCl added, they were neutralized and otherwise treated as described above. The concentrations in the analyses were 0.04, 0.08 and 0.12 mg/26 ml, and for each of these concentrations 6—8 separate analyses were made. The averages for each concentration are presented in the form of a curve in Fig. 3.

The reproducibility of the method is excellent. The author has employed the method for over a year and has found it reliable on condition that pure reagents are used and that the standard concentrations, reaction times and temperatures are strictly maintained.

Table III. *Analyses after acetylation at 95 and 100° C.*

Number of analyses	Acetylation temperature in °C	Optical density
5	95	0.354
5	100	0.352

Table IV. *Analyses with and without the addition of lysin HCl and glucose.*

0.1 ml of:	Optical density
0.0056 M glucosamine HCl	0.172
0.0056 M glucosamine HCl +0.093 M glucose+0.0093 M lysine HCl	0.176

Normal material

This consists of sera from 57 healthy blood donors aged 18 to 61 years, of which 26 women and 31 men. The results of the investigations are recorded in Table V and fig. 4. The values obtained are in good agreement with those reported in the literature: Björnesjö (1954) 98 mg/100 ml, Palmer *et al.* (1937) 90—130 mg/100 ml, Stary (1950) 96 mg/100 ml, West & Clarke (1938) 76—110 mg/100 ml. The absolute glucosamine values are correlated with the total protein values (determined by the Biuret method) and are recorded in the table (glucosamine/TP · 100). This correlation is important, and total protein should always be determined parallel to glucosamine determination, as large variations in the fluid balance would otherwise give seemingly large variations in the glucosamine concentration.

SUMMARY

A brief description is given of the principle for hexosamine determination according to Pauly & Ludwig and Elson & Morgan and of Blix' and Sundblad's modifications of the method. Some of the steps in the metho

Table V. *Serum glucosamine determinations in normal material, 57 healthy blood donors aged 18—61 years.*

Sex	Number	Total protein in per cent		Glucosamine mg/100 ml		Glucosamine in per cent of total protein	
		Mean	SD	Mean	SD	Mean	SD
Male.....	31	7.00	0.37	98	8	1.40	0.11
Female	26	6.72	0.33	98	10	1.46	0.12
M+F.....	57	6.87	0.36	98	9	1.43	0.12

SD = Standard deviation of the mean.

are analysed in more detail, and the introduction of direct titration in the analysis after hydrolysis is suggested with bromthymol blue as indicator. It has been shown experimentally that this indicator does not influence the analytical result. The significance of impure acetyl acetone as a possible source of error is pointed out. Some slight modifications are described. It is shown experimentally that glucose-lysine has no significant influence on the analytical results in this method. The author's modification of the method is described, and the result of examination of 57 normal sera is presented. The importance of correlating the glucosamine values with total protein values is emphasized. The method is applicable to other biological materials containing hexosamine.

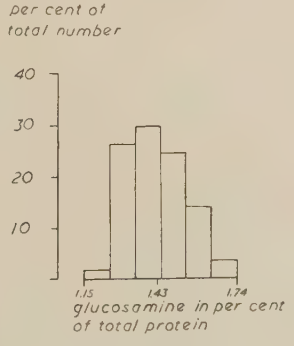

Fig. 4. Range of normal values for glucosamine/-total protein ratio in 57 healthy blood donors.

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MEASUREMENT OF CORONARY BLOOD FLOW AND CARDIAC EFFICIENCY IN HYPOTHERMIA BY USE OF RADIOACTIVE KRYPTON 85

By A. TYBJÆRG HANSEN, B. F. HAXHOLDT, E. HUSFELDT, N. A. LASSEN, O. MUNCK, H. RAHBEK SØRENSEN and K. WINKLER

From Department of Medicine B, Cardiovascular Laboratory and Department of Surgery D, Rigshospitalet, Copenhagen

(Received for publication March 15, 1955)

The demonstration that animals in hypothermia can withstand much longer periods of exclusion of the heart from the circulation than animals at normal body temperature (Bigelow, Callaghan & Hopps, 1950) has made physiologic studies of the effect of cooling important. Recent observations made in dogs by use of the nitrous oxide desaturation method (Edwards, Tuluy, Reber, Siegel & Bing, 1954) demonstrated that coronary blood flow and myocardial oxygen consumption decreased to approximately 55 per cent of control values at a body temperature of 26—27°C. Coronary arteriovenous oxygen difference remained unchanged. Furthermore a proportionally greater fall inn cardiac work than in myocardial oxygen uptake suggested a failure of the mechanical efficiency of the heart in the cold state.

The present investigation was undertaken to determine the circulatory and oxygen metabolic adjustments of the heart during various stages of hypothermia.

METHODS

Five healthy dogs, varying in weight from 22 to 33 kg, were used. The animals were anesthetized with nembutal sodium given intravenously in a dosage of approximately 30 mg per kg body weight. Manually controlled respiration was started after tracheal intubation and maintained throughout the procedure. The heart was exposed after left-side thoracotomy, and polyethylene tubes were placed in the coronary sinus, in the pulmonary artery, and in the aorta. The tube in the coronary sinus was placed as far from the auricular ostium as possible in order to avoid contamination with systemic venous blood. Each of the tubes was connected to manifolds for withdrawal of blood samples; for measuring the mean arterial blood pressure a mercury manometer was used.

Coronary blood flow was measured by the krypton 85 method (Lassen & Munck, 1955) with the modification that the blood samples were taken during desaturation in order to avoid errors due to possible leaks around the cuffed treachael tube. The krypton 85 method is based on the same principle as the nitrous oxide technique, *viz.* the exchange of an inert gas between blood and myocardial tissue. For each flow determination the dogs inhaled atmospheric air containing radioactive krypton 85 for approximately 14 minutes. At the end of the saturation period arterial and coronary sinus blood was obtained for determination of krypton 85 at the saturation level. During the following 10—18 minutes of desaturation 5 blood samples were collected simultaneously from the aorta and the coronary sinus, so that a total of 12 samples were obtained. The procedures employed in analyzing the samples for radioactivity have been described elsewhere (Lassen & Munck, 1955). Samples were taken for the determination of arterial coronary sinus, and systemic venous oxygen content (Van Slyke & Neill, 1924) and for the measuremtnt of arterial pH, carbon dioxide tension and total carbon dioxide content (Astrup, 1956).

Cardiac output was estimated by the direct Fick method (Fick, 1870) immediately before each krypton 85 saturation period. Carbon dioxide and oxygen in expired air were measured with the Haldane apparatus (Haldane & Priestly, 1935). Body temperature was measured with a rectal and esophagal thermocouple. In case of discrepancy esophagal temperature was used.

This series of measurements was carried out three times in each animal, at approximately 37° C, 28° C, and 23.5° C.

Cooling was undertaken with ice bags. Small supplementary doses of nembutal (app. 7 mg/kg) were given intravenously to each dog during the initial phase of cooling in order to prevent shivering. At least one hour elapsed between supplementation of anesthesia and sampling of blood for the measurements of coronary flow and myocardial metabolism. The three periods of measurements were conducted at about the same, very light, anesthetic level, except for the effect of cold. 400—500 ml of blood were given during the experiment to cover the blood loss from operation and blood sampling.

CALCULATIONS

Coronary blood flow was calculated from curves representing the changing concentrations of krypton 85 in arterial and coronary sinus blood during the desaturation period (Fig. 1) using the formula of Kety & Schmidt (1948) except for differences arising from the use of desaturation curves. A partition coefficient blood : myocardium of 1 was used, as the relative solubility of krypton 85 was assumed to be similar to that of nitrous oxide (Eckenhoff, Hafkenschiel, Harmel, Goodale, Lubin, Bing & Kety, 1947 a). As coronary sinus blood is considered chiefly to drain the left ventricular muscle (Gregg & Shipley, 1947), the blood flow obtained was taken to represent left ventricular myocardial perfusion. The blood flow measured by inert gas diffusion technique expresses the perfusion rate of a unit of tissue, usually 100 g, and coronary blood flow is accordingly given in cc/100 g/min. Blood flow for the entire left ventricular musculature could be calculated as the actual weight of the left ventricle was determined *post mortem* in all animals. Left ventricular oxygen consumption was

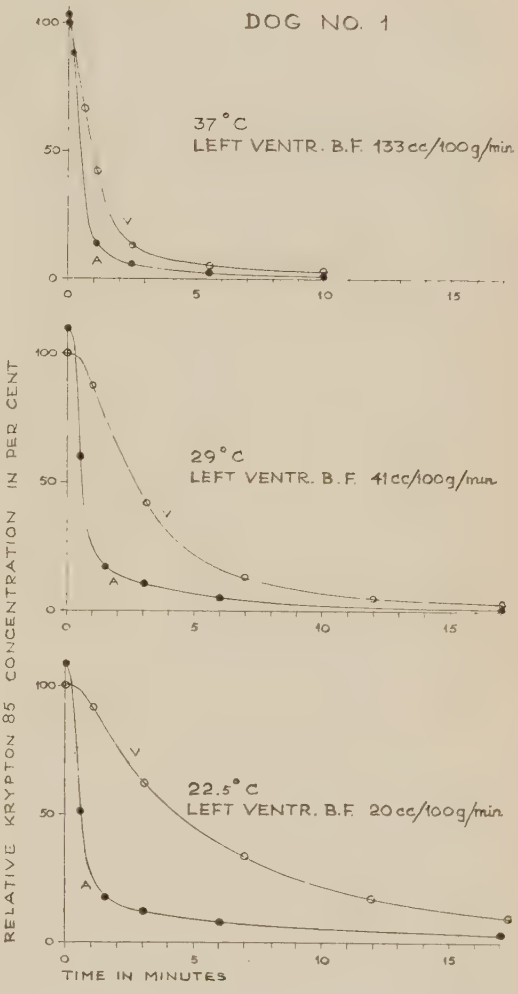

Fig. 1. Arterial (A) and coronary venous (V) krypton 85 desaturation curves at different temperature levels in dog No. 1. Coronary blood flow (LEFT VENTR. B.F.) is given in cc/100 g myocardial tissue/min.

calculated by multiplying the coronary blood flow per gram tissue per minute with the coronary arteriovenous oxygen difference.

Cardiac output was calculated by the Fick principle. As approximately 20 minutes elapsed between sampling for the measurement of cardiac

Table I. *Circulatory functions in hypothermia.*

No.	Weight kg	Left ventricle weight g	Temp. °C	Pulse rate	Mean aortic B. Pr. mm Hg	Coronary blood flow cc/100 g/min.	Total coronary blood flow of left ventricle cc/min.	Cardiac output cc/min.	Stroke vol. cc/beat	Total coronary blood flow, of left ventricle, in per cent of cardiac output
1	32.5	143	37.0	188	93	133	190	3500	19	5.4
			29.0	100	83	41	59	1280	13	4.6
			22.5	44	78	20	29	800	18	3.6
2	23.0	121	37.5	136	105	88	107	2030	15	5.3
			27.0	74	73	33	40	920	12	4.4
			22.0	41	70	18	22	660	16	3.3
3	21.5	113	38.0	150	65	48	54	2340	16	2.3
			29.0	88	53	14	16	730	8	2.2
			25.0	55	32	9	10	530	10	1.9
4	30.0	120	37.3	152	111	85	102	2660	18	3.8
			32.0	140	119	100	120	2680	19	4.5
			25.0	64	84	36	43	1050	13	4.1
5	22.5	110	36.0	166	86	85	94	2840	17	3.3
			27.5	100	93	47	52	1380	14	3.8
			24.5	72	65	32	35	900	13	3.9

output and the determination of coronary flow, interpolation of cardiac output values to the time of flow measurements was made, as a difference in time of 20 minutes might mean a difference in temperature of 2—4° C.

Left ventricular work in kg meter/min. was calculated as: cardiac output (kg/min.) × mean aortic pressure (meters Hg) × 13.6. No allowance was made in this calculation for the velocity energy, since at rest this component represents less than 5 per cent of the left ventricular work (Evans & Matsuoka, 1914). Total left ventricular energy cost was calculated in the same units as the left ventricular work by multiplying the oxygen uptake of the total left ventricle by the work equivalent of oxygen at a tissue respiratory quotient of 0.82 (2.059) (MacLeod, 1926). The mechanical efficiency of the left ventricle was calculated as the percentage of total left ventricular energy cost which was converted to pressure work: Efficiency = 100 × left ventricular work/total left ventricular energy cost.

RESULTS

The effects of hypothermia on the circulatory functions studied are shown in Table I. The coronary blood flow and the cardiac output decreased proportionally. This demonstrates that the coronary circulation accounts for a relatively constant fraction of the cardiac output: approximately 3.5 per cent of the cardiac output perfuses the left ventricular musculature. One observation made during shivering in dog No. 4 at 32° C failed to show decreased values.

Table II shows the oxygen metabolism in hypothermia. The systemic and coronary arteriovenous oxygen difference remained essentially unchanged. As myocardial energy cost and cardiac work showed a proportional

Table II. *Oxygen metabolism in hypothermia.*

No.	Weight kg	Temp. °C	Arteriovenous oxygen diff. Systemic cc 100 cc	Arteriovenous oxygen diff. Coronary cc/100 cc	Total body O_2 consump. cc/min.	Total left ventr. work kgm/min.	Left ventr. O_2 consump. cc/100 g/min.	Total left ventr. O_2 consump. cc/min.	Total ventr. energy cost kgm/min.	Mechanical efficiency in per cent	L.V. O_2 uptake in per cent of total O_2 uptake in per cent
1	32.5	37.0	5.1	14.5	178	4.42	14.5	20.8	42.0	11	12
		29.0	6.1	11.7	78	1.44	4.8	7.0	14.4	10	9
		22.5	5.9	13.2	47	0.85	2.6	3.8	7.8	11	8
2	23.0	37.5	7.0	11.0	142	2.90	11.0	13.4	27.6	11	9
		27.0	6.4	12.1	59	0.91	4.0	4.8	9.9	11	8
		22.0	6.5	11.2	43	0.63	2.0	2.5	5.1	12	6
3	21.5	38.0	7.2	13.9	169	2.07	6.7	7.6	15.6	13	5
		29.0	10.8	16.3	79	0.53	2.2	2.5	5.1	10	6
		25.0	11.4	15.3	60	0.23	1.4	1.5	3.1	7	5
4	30.0	37.3	6.7	13.0	178	4.02	11.5	13.8	28.4	14	8
		32.0	7.2	12.0	193	4.34	12.0	14.4	29.7	14	7
		25.0	5.5	14.8	58	1.20	5.3	6.4	13.2	9	11
5	22.5	36.0	5.7	13.2	162	3.32	11.2	12.3	25.4	13	8
		27.5	7.1	14.9	98	1.74	7.0	7.7	15.9	12	8
		24.5	7.1	14.6	64	0.72	4.7	5.2	10.7	7	8

Table III. *Pulmonary functions in hypothermia. The total body respiratory quotients are also given.*

No.	Weight kg	Temp. °C	Oxygen capacity cc/100 cc	Arterial O_2 saturation in per cent	Arterial pH	Arterial pCO_2 mm Hg	Arterial total CO_2 mEq/l	Ventilation l/min.	RQ
1	32.5	37.0	19.1	92	7.426	32	20.8	3.46	0.62
		29.0	19.0	92	7.402	30	18.5	2.38	0.70
		22.5	19.9	93	7.348	30	17.8	3.84	0.60
2	23.0	37.5	18.2	94	7.437	30	20.0	3.43	0.58
		27.0	18.3	95	7.461	24	16.9	3.55	0.58
		22.0	18.4	97	7.440	24	16.1	5.19	0.76
3	21.5	38.0	20.5	94	7.449	27	18.5	12.65	0.89
		29.0	19.9	94	7.339	31	16.7	8.15	0.68
		25.0	19.7	92	7.461	18	12.7	5.43	0.67
4	30.0	37.3	17.9	92	7.540	13	8.7	5.47	0.43
		32.0	23.3	73	7.341	28	15.1	4.08	0.44
		25.0	27.9	80	7.480	11	7 8	4.32	0.61
5	22.5	36.0	21.2	91	7.581	13	11.6	7.13	0.57
		27.5	23.0	92	7.621	10	9.8	6.82	0.53
		24.5	25.3	95	7.661	8	8.6	5.31	0.77

decrease during hypothermia, no significant alterations were observed in mechanical efficiency, which in the normothermic state averaged 12 per cent (11—14 per cent), at 28° C 12 per cent (10—14 per cent), and at 23.5° C 9 per cent (7—12 per cent).

Some data mainly referring to pulmonary function in hypothermia are given in Table III. The controlled ventilation was efficient in maintaining arterial oxygen saturation, pH, and carbon dioxide tension, except in dog No. 4, which had been subject to hypoventilation in the cold state. The total body respiratory quotient was low in all cases and unaffected by cooling.

DISCUSSION

The application of the krypton 85 method for the measurement of coronary blood flow and myocardial oxygen consumption gave control values similar to those obtained with bubble flow meter and with the nitrous oxide method in anesthetized dogs (Edwards *et al.*, 1954; Eckenhoff *et al.*, 1947 a, 1947 b, 1948; Harrison, Friedman & Resnik, 1936; Goodale, Lubin, Eckenhoff, Hafkenschiel & Banfiel, 1948). Only the control series of Edwards *et al.* (1954) deviates significantly from the others, as their values for coronary blood flow and oxygen consumption were twice the value found by other investigators.

The values for cardiac efficiency were lower in our series than those generally appearing in the literature, but this it not remarkable in view of the rater low values for cardiac output and mean aortic blood pressure. Eckenhoff *et al.* (1948) studied the influence of hypotension on cardiac efficiency, and at a blood pressure level similar to our control values the cardiac efficiency averaged 15 per cent as compared to 12 per cent in the present study. In their excellent discussion these authors concluded that the decline in cardiac efficiency from 36 per cent to 15 per cent induced by hypotension offered no indication of the heart being less able to accomplish the work demanded of it.

The present investigation demonstrated that cooling to approximately 28° C effected a considerable decrease in coronary blood flow, left ventricular oxygen uptake, cardiac output, cardiac work, and total body oxygen uptake to about 1/3 of control values. By cooling to approximately 23.5° C the same functions were reduced to about 1/4 of control values. Several investigators have reported on cardiac output in hypothermia (Edwards *et al.*, 1954; Bigelow *et al.*, 1950; Prec, Rosenman, Braun, Rodbard & Katz, 1949), and our results agree favorably with their findings.

By cooling to approximately 26° C, Edwards *et al.* (1954), who used the nitrous oxide technique and percutaneous heart catheterisation, found a decrease in cardiac efficiency from 20 per cent to 8 per cent, and they suggested a mechanical failure of the heart in the cold state. From the nine experiments at a temperature below 30° C (average 26° C) in the present study a mean value for cardiac efficiency of 10 per cent can be calculated, a value not significantly lower than the 12 per cent found at normal body temperature. This discrepancy presumably reflects the greater reduction in mean aortic blood pressure from 143 mm Hg to 74 mm Hg in the nitrous oxide series compared to the fall from 92 mm Hg to 67 mm Hg in the present study. Thus it is tempting to assume that the reduction in

cardiac efficiency demonstrated by Edwards *et al.* (1954) was not caused by hypothermia *per se* but by the concomitant hypotension, a suggestion which is supported by the above cited findings during induced hypotension (Eckenhoff, 1948).

From experimental cardiac surgery it is established that at very low temperatures the risk of fatal ventricular fibrillation is high. Recently it has been stated from clinical observations (Bigelow, 1955; Swan, 1955) that at a temperature level of 28—30° C the incidence of ventricular fibrillation was only insignificantly higher than at normal temperature. This clinical experience and our laboratory findings suggest that a moderate cooling to 28—30° C, which reduces tissue oxygen demand to 40—50 per cent, could be of considerable aid in cardiac surgery without involving any noticeably increased risk of ventricular fibrillation.

SUMMARY

A method has been described for the measurement of coronary blood flow by use of radioactive krypton 85. Control values for coronary blood flow and myocardial oxygen consumption agreed with results obtained in the anesthetized dog by previous investigators, who used the bubble flow meter or the nitrous oxide technique.

Observations of coronary blood flow and myocardial oxygen metabolism were performed in dogs at two levels of hypothermia induced with ice bags. Coronary arteriovenous oxygen difference and cardiac mechanical efficiency remained essentially unchanged from the normothermic state. Coronary blood flow, left ventricular oxygen consumption, cardiac output, cardiac work, and total body oxygen uptake showed a proportional decrease to 1/3 of control values at 28° C and to 1/4 at 23.5° C. No indication was found of the heart being less able to accomplish the work demanded of it during hypothermia.

From the clinical experience reported in the literature and the present laboratory data it was concluded that cooling to 28—30° C would be of considerable aid in cardiac surgery without involving any noticeably, increased risk of ventricular fibrillation.

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THE USE OF BROMSULPHALEIN FOR DETERMINATION OF THE CARDIAC OUTPUT

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T-1824, Evans Blue, the most extensively used dye for measuring the cardiac output by dye dilution techniques, is easy to determine in plasma. However, it does not permit more than two consecutive measurements a day due to discoloration of the skin. Vidt, Hanusek, Schieve, Hull & Sapirstein (1955) reported the use of Rose Bengal (tetraiodo-tetrabrom-fluorescin) to determine cardiac output in dogs. This dye is eliminated rapidly from the blood, but is somewhat difficult to determine, special precautions being necessary to avoid hemolysis due to its photodynamic effect. For these reasons it was considered worth while to look for another substance which does not color the skin, or cause other disagreable symptoms.

This paper describes the use of Bromsulphalein for determination of the cardiac output. The results obtained with this dye have been compared with those obtained from Evans Blue injected simultaneously.

MATERIAL

A total number of 16 determinations were made on 3 healthy subjects and on 11 patients subjected to heart catheterization for the purpose of hemodynamic studies.[1]

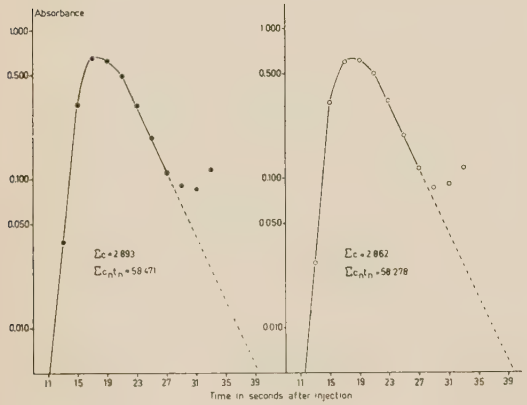
METHODS

The technique for determination of the cardiac output by the dye dilution method is essentially that of Hamilton, Moore Kinsman, & Spurling (1931—32); Hamilton, Riley, Attyah, Cournand, Fowell, Himmelstein, Noble, Remington, Richards, Wheeler & Witham (1948) as modified by Lagerlöf, Bucht, Werkö, & Holmgren (1949).

About 4 ml each of Evans Blue dye (T-1824) 0.5 per cent and Bromsulphalein (B.S.P.) 5 per cent are thoroughly mixed in a 10 ml syringe calibrated to deliver exactly 8 ml. In these experiments as in those of the above mentioned authors the dyes were injected through a Cournand catheter. (If one wants to use B.S.P. alone only 4 ml of a 5 per cent solution is injected.) In most of the experiments the tip of the catheter was placed in the main stem of the pulmonary artery.

Prior to the injection of dye a blood sample is taken to be used as a blank. The dye mixture is then rapidly injected and at the same time the collection of blood samples is started from an indwelling needle in one of the brachial arteries. The blood is collected in previously heparinized tubes, about 10 × 50 mm, placed in a rack which is moved by hand so that a blood sample is obtained every other second. About 0.5—1 ml of blood is collected in each tube. The dye solution left in the catheter is immediately withdrawn into the syringe. The syringe is emptied and rinsed in about 50 ml 0.9 per cent NaCl. This solution is then diluted to 100 ml and centrifuged. 5 ml of the

[1] Most of the patients were from the IIIrd Medical Service of this hospital. The head of the service, Docent Ebbe Nyman, has kindly placed the material at my disposal. I am indebted to Docent Lars Werkö and his colaborators for valuable help with the catheterization.



Experiment 3 A. Injection at 0 time. Collecting of blood samples started 5 seconds after the injection. Left Evan Blue, right B.S.P.

supernatant is further diluted by mixing with 5 ml of pooled serum without dye and M/15 phosphate buffer pH 6.8 to 50 ml. The absorbance of this solution is read against a blank made in the same manner but omitting the dye. Correction is made for the dyes left in the catheter and the syringe in calculation of the injected amounts of dyes. The ratio between the two dyes in the withdrawn fluid is used in calculation of the total amounts injected.

The arterial blood samples are centrifuged and the plasma is transferred to microcuvettes. The absorbance of Evans Blue is read in a photoelectric colorimeter (Coleman Jr. or a Beckman DU with microcell attachment) at 620 mμ using undyed plasma as a blank. On standing the plasma becomes alkaline which makes the B.S.P. colour appear and interfere with the reading of the Evans Blue. This can be overcome by blowing CO_2 on to the surface in the cuvette for a few seconds immediately before the reading and by stirring the content with a thin glass rod. Care has to be taken to avoid foaming. This operation is omitted when the withdrawn solution is measured.

The B.S.P. content is determined after dilution of 0.1 ml plasma with 2.9 ml 0.1 N NaOH. The absorbance is read at 580 mμ against a blank containing 0.1 ml of the same plasma and 2.9 ml 0.1 N HCl. Fortunately the wavelength 580 mμ happens to be both the absorption maximum of B.S.P. and the isobestic point for Evans Blue in acid and alkaline solution. Thus the influence of the Evans Blue color is eliminated by the use of an acid blank when the B.S.P. color is read at this wavelength.

Samples with readings over 0.600 are diluted 1:2 with undyed plasma in case of Evans Blue and 1:60 with 0.1 N NaOH in case of B.S.P.

CALCULATIONS

All results are plotted on semilogarithmic paper and extrapolated to infinity according to Hamilton *et al.* (1948, see above). The ordinate represents the concentrations and the abscissa time in seconds. Cardiac output (CO) is calculated according to the formula:

$$CO = \frac{60 \cdot F \cdot 100}{2\Sigma c \cdot (100 - H)}$$

CO = Cardiac output in liter/min.
F = ml dye solution injected.

F is calculated according to the formula:

$$F = \frac{W_1 \cdot 8}{W_1 + W_2} - W_1 \text{ or } W_2 \text{ resp.}$$

W_1 and W_2 represent the volume of original solution withdrawn recalculated as if it were 0.5 per cent Evans Blue and 5 per cent B.S.P. When B.S.P. is used only 4 ml is injected and F has to be calculated according to the formula:

$$F = 4 - W.$$

C = sum of concentrations determined every other second in the plasma from the brachial artery. C is expressed as ml dye solution/liter plasma after graphical deduction of the recirculation.

H = hematocrit value.

The volume (V) passed by the dye, catheter tip to artery cannula, is given by the formula:

$$V = \frac{CO}{60} Tm$$

where tm is the mean circulation time in seconds.

$$Tm = \frac{c_1 \cdot t_1 + c_2 \cdot t_2 \ldots + c_n \cdot t_n}{c} + K$$

Table I.

No.	Diagnosis	Cardiac output in l/min. (CO)		Mean circulation time in sec. (Tm)		Volume injection site — Br. Art. in liter (V)		
		Ev. Blue	B.S.P.	Ev. Blue	B.S.P.	Ev. Blue	B.S.P.	Injection site
1	Cough syndron	9.89	8.84	11.29	11.36	1.86	1.67	Pulm.Art.
2 A	Hypertension	6.02	5.98	10.29	10.09	1.03	1.00	—»—
2 B*	Hypertension	7.23	7.41	8.46	8.56	1.02	1.06	—»—
3 A	Healthy	6.25	6.16	20.46	20.61	2.13	2.12	Renal Vein
3 B*	Healthy	10.41	10.31	13.41	13.58	2.33	2.33	—»—
4	Cough syndrom	5.53	4.82	13.36	13.62	1.23	1.09	Pulm.Art.
5	Hypertension	5.77	6.64	21.02	19.89	2.02	2.00	Brachial Vein
6	Tricuspidal stenosis	4.30	4.73	14.21	14.42	1.02	1.14	Pulm.Art.
7	Mitral stenosis	6.17	7.37	10.63	10.64	1.09	1.31	—»—
8**	Nephrosis	(5.15	7.24	18.18	17.48	1.56	2.11)	Renal Vein
9	Healthy	7.11	8.23	13.86	14.11	1.69	1.80	Pulm.Art.
10	Heart neurosis	7.04	7.04	14.19	14.22	1.66	1.67	—»—
11	V.O.C. mitral	4.84	5.41	12.12	12.91	0.98	1.16	—»—
12	Healthy	6.23	6.00	12.24	12.27	1.27	1.23	—»—
13	Hypertension	6.50	7.00	15.34	15.27	1.66	1.78	—»—
14	Mitral	3.97	3.45	19.21	19.17	1.27	1.10	—»—
	Mean	6.484	6.626	14.006	14.048	1.479	1.497	
	Standard deviation	1.771	1.736	3.690	3.531	0.450	0.437	
	Standard error	0.457	0.448	0.912	0.953	0.116	0.116	
	Mean diff. B.S.P.—Ev.Blue	0.142±0.640		0.042±1.319		0.018±0.162		
	t	0.222		0.032		0.111		
	p	<0.9>0.8		<1.0>0.9		<1.0>0.9		

* Results obtained during rapid intravenous infusion of large amounts of isotonic dextran solution.
** This case had a lipemic serum with varying turbidity in the different samples which gave an uneven curve for Evans Blue. It is therefore not included in the statistical calculation.
Results of the determination of the cardiac output with Bromsulphalein and Evans Blue used simultaneously.

where c_n is the concentration t_n seconds after the injection. K is a constant for the time which it takes for the dye to pass through the catheter and is approximately estimated to 0.25 seconds.

CALIBRATIONS

The B.S.P used in all the determinations was from one lot as was the Evans Blue. For the sake of convenience all calibrations were done in ml of dye solution/liter. Since the color of Evans Blue changes in the presence of protein, the absorbance of this dye was standardized by reading of standard solutions diluted with a mixture of 9 volumes 0.9 per cent NaCl and 1 volume of pooled serum without dyes. In all B.S.P. determinations the plasma was diluted with 0.1 N NaOH. The presence of serum protein is without effect on the absorbance of B.S.P.

RESULTS

The results are summarized in Table I.

CONCLUSIONS AND SUMMARY

As the B.S.P. and the Evans Blue dye are given in the same solution, spontaneous variations in the cardiac output do not interfere when comparing the results obtained with the two dyes. There does not seem to be any systematic difference between the results obtained with the two substances. Thus the dyes not seem to be of influence on each other's determinations. Further the agreement between the results show that there is no loss of B.S.P. due to diffusion into the lungs during the first circulation as is the case with, for instance, phenoltetraiodophtalein sodium. (Hamilton, Walker, Moore, Kinsman, & Spurling, 1931/32; Hamilton & Remington, 1947). Both the dyes are strongly bound to the albumin. The dissociation constants of the dye-protein complex is 2×10^{-6} for Evans Blue (Allen & Orahovats, 1948; LeVeen & Fishman, 1947) and 7×10^{-6} for B.S.P.

The advantage of B.S.P. is that it can be used in serial determinations of the cardiac output without discoloration of the skin. As the plasma is diluted 30 times, errors due to opalescence and discoloration of the plasma is of much less importance when B.S.P. is used than with Evans Blue which is usually read without dilution of the plasma.

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CARDIAC OUTPUT, BLOOD PRESSURES AND RENAL DYNAMICS IN COARCTATION OF THE AORTA

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The renal dynamics in patients with coarctation of the aorta have usually been compared with the findings in arterial hypertension in an attempt to establish — or rule out — a causal relationship between decreased renal blood flow and the occurrence of hypertension in patients with coarctation of the aorta. In a large series of different heart lesions it was demonstrated that the renal blood flow was diminished but without alteration in filtration rate, findings parallel to those found in arterial hypertension. (Werkö, Ek, Varnauskas, Thomasson & Lliash, 1955). It thus seemed of interest to study patients with coarctation of the aorta with regard to their kidneys and compare the results to those obtained in congenital or rheumatic heart lesions.

The present report contains the findings of renal and hemodynamic studies in 15 patients with coarctation of the aorta, 4 of them studied during rest and exercise and 7 of them before and after operation for the aortic lesion.

* Aided by grants from the Swedish Medical Research Council and the Knut and Alice Wallenberg Foundation. Presented in part at a meeting of the Swedish Society for Cardiology. December 1955.

MATERIAL

Fifteen patients with coarctation of the aorta were studied. Four of the patients had other heart lesions also, all of them aortic incompetence, two of them also had signs of ventricular septal defect and one mitral valve disease. All were referred for study before operation. Seven of the patients were again studied after the coarctation had been surgically corrected. In one patient the renal clearances and intra-aortic blood pressure were again determined one year after operation.

METHOD

All studies were performed in the morning with the subject recumbent and in the postabsorptive state, except for one-half liter of plain water given perorally before the start of the procedure.

The pulmonary artery was catheterized according to Cournand (1941) and an indwelling arterial needle placed in the brachial artery. Cardiac output was determined according to the direct Fick principle with simultaneous sampling of blood from the pulmonary and brachial arteries and expired air. The blood gases were determined on a Van Slyke apparatus and the air gases on a Haldane apparatus. Blood pressures in the pulmonary and systemic circuit were registered with Tybjærg-Hansen electrical capacitance manometers.

Resting values for cardiac output and blood pressures were obtained after more than half an hour's rest after the catheter had been placed in the pulmonary artery.

The renal clearances for inulin, endogenous creatinine, and para-amino-hippurate were determined simultaneously, in some patients after a single in-

travenous injection of inulin and a single intramuscular injection of para-amino-hippurate. In other patients, these substances were given intravenously with a constant-speed injection syringe. Urine was collected at 10 or 15 minute intervals through an indwelling catheter, and the bladder was rinsed twice with distilled water and air after each urine collection. Resting clearance values were obtained during two-three periods. The last resting clearance period was always completed shortly after the determination of the resting cardiac output.

The details of the procedure and method of determinations of the test substances have been published before (Werkö *et al.*, 1955).

After the resting determination, some patients performed a graded amount of exercise, recumbent, on a bicycle ergometer. The clearance values, cardiac output, and blood pressures were again determined during at least 10 to 15 minutes of exercise.

In one patient determinations were repeated several times during the intravenous infusion of isotonic dextran solution at a rate of about 25 ml/min.

Seven patients in the present series were studied again after the heart lesion had been corrected by surgical treatment. The patients were operated on in the Surgical Department, Sabbatsberg Hospital.

When aortography was performed the blood pressures in the aorta were recorded above and below the stenosis.

RESULTS

Table I contains the figures for cardiac output, arteriovenous oxygen difference, blood pressures in the pulmonary and systemic circulation and inulin and para-amino-hippurate clearance in 11 patients with uncomplicated coarctation of the aorta and four patients with coarctation complicated with heart disease. In the first group the pressures in the pulmonary circuit were normal, in the second sligthly elevated. The blood pressure fall over the coarctation ranged between 9 and 54 mm Hg (determined directly). The para-amino-hippurate clearance was between 316 and 694 ml/min. and tended to be slightly lower in the second group. The inulin clearance ranged between 89 and 173 ml/min. and was the same in both groups.

Table II contains the same values during rest ond in some patients during exercise before and after operation. The para-amino-hippurate clearance was unaltered in three patients and increased in four after operation, giving a moderate increase in the group as a whole.

The reaction to exercise was unaltered by operation, with small changes in renal blood flow, filtration rate and sodium excretion. This is also illustrated in Fig. 1.

In Fig. 2 the filtration rate and renal plasma flow are correlated to the pressure above and below the stenosis in case G-r before, 2 months after and 16 months after the operation. Renal blood flow decreased slightly, concomitant to the fall in arterial pressure immediately after operation, but increased to normal values when the arterial pressure rose to hypertensive level. There was no apparent correlation to the amplitude of the aortic pressure.

Table III shows a comparison between the renal findings in the present series and in certain other heart lesions with moderate decrease of function. The para-amino-hippurate clearance in uncomplicated coarctation was about the same as in patent ductus arteriosus and atrial septal defect. In the patients with heart disease complicating the coarctation the para-amino-hippurate clearance was lower and more comparable to the findings in patients with mitral valvular disease in functional groups I—II.

Table I. *Renal clearances, cardiac output and blood pressures in a) 11 patients with uncomplicated coarctation of the aorta and b) 4 patients with coarctation of the aorta complicated with valvular heart disease.*

Case No	Sex	Age	BSA m^2	Pulse rate	O_2 consump. ml/min	A-V O_2 diff. ml/l	Cardiac output l min	Pressures in mm Hg				Clearance in ml/min.	
								RA	PA	BA	Decrease over the stenosis	PAH	Inulin
								M	M	M	M		
580	F	21	1.60	71	241	41	5.93	0	10	90	9	347	110
718	F	49	1.77	70	177	55	3.22	-2	8	82	—	299	147
760	M	24	2.02	133	369	38	9.76	—	17	122	29	—	—
779	M	30	1.73	102	328	32	10.40	0	12	121	28	694	176
798	F	31	1.80	83	160	33	4.91	0	10	128	—	316	95
799	M	21	1.87	87	268	38	7.10	0	7	90	25	540	99
865	M	29	1.87	69	339	51	6.71	4	15	122	23	666	—
					322		6.34						
G-n	M	21								91	21	557	108
E-n	M	30								123	54	530	98
C-n	M	21								112	27	575	114
G-r	M	19								147	25	522	126
n							8	6	7	7		10	9
Mean							6.80	0.3	11	108		505	119
653	F	25	1.64	86	215	29	7.34	1	16	105	33	495	173
776	M	28	1.58	75	330	52	6.38	1	31	92	10	378	136
819	M	22	1.54	77	267	31	8.67	—	15	89	31	406	89
820	M	28	1.72	72	292	37	7.96	—	18	105	47	457	108
n							4	2	4	4		4	4
Mean							7.59	1	20	98		434	127

Fig. 3 demonstrates the reaction to a rapid infusion of dextran in a case of coarctation. The renal plasma flow increased markedly (from about 650 to 1050 ml/min.) in response to the expansion of the plasma volume, without any change in arterial blood pressure and with only little alteration of the cardiac output.

DISCUSSION

The elevation of the blood pressure in coarctation of the aorta has attracted considerable attention especially since the lesion became amenable to surgical correction. Much discussion has been based on single blood pressure determinations and in elderly patients. In the present series, consisting of mostly younger persons, the blood pressure

Table II. *Pre- and postoperative studies in a) 5 patients with uncomplicated coarctation of the aorta and b) 2 patients with coarctation of the aorta complicated with valvular heart disease.*

Case No	Time	Pressures in mm Hg			Cardiac output l/min.	Clearance in ml/min.		Na+ excret. uEq/min.
		RA M	PA M	BA M		PAH	Inulin	
580 preop.	R	0	10	90	5.93	347	110	259
	E	0	14	99	7.72	364	112	114
postop.	R					750	84	—
799 preop.	R	0	7	90	7.10	540	99	86
	E	-2	16	99	8.18	596	107	22
postop.	R			81		507	136	163
	E			90		458	143	112
G-n preop.						557	108	
postop.						735	115	
E-n preop.						530	98	
						523	123	
G-r preop.						522	125	380
postop.						463	106	
n preop.		0	2	2	2	5	5	3
M R		0	9	90	6.52	499	108	242
n postop.						5	5	
M R						596	113	
819 preop.	R	—	15	89	8.67	406	89	141
	E	-1	18	98	7.60	456	96	137
862 postop.	R	1	14	109	6.34	108	108	300
	E	-1	22	109	7.64	450	108	223
820 preop.	R	—	18	106	7.96	457	108	97
	E	-4	26	110	11.60	437	124	71
870 postop.	R	2	16	83	6.59	749	181	210
	E	-3	20	82	11.31	740	228	206
n preop.		—	2	2	2	2	2	2
M R			17	97	8.32	432	99	119
n preop.		2	2	2	2	2	2	2
M E		-2.5	22	104	9.60	447	110	104
n postop.		2	2	2	2	2	2	2
M R		1.5	15	96	6.47	635	145	255
n postop.		2	2	2	2	2	2	2
M E		-2	21	96	9.48	595	168	215

Table III. *Blood pressures, cardiac output and renal clearances in 63 caces with different heart disorders.*

Diagnosis	No Case	Cardiac output in l/min.	Blood pressures in mm Hg			Clearance in ml/min.	
			Right Atrium	Pulm. Artery	Brach. Artery	PAH	Inulin
atent ductus arteriosus	8	6.6	1	14	89	473	123
uricular septal defect	7	12.6	1	12	75	502	126
itral valvular disease group I—II ...	26	6.2	1	20	91	405	109
rtic valvular disease group I—II ...	9	7.7	1	14	89	480	138
oarctatio aorta	9	6.8	0	11	108	503	118
oarctatio aorta + V.O.C.....	4	7.6	1	20	98	434	127

all patients except one was normal or close normal in the upper part of the body, with e same mean pressure and considerably ecreased pulse pressure in the lower part the body. In this series the blood pressure us does not show much in common with e blood pressure arterial hypertension. The ssimilarity of the blood pressure in coarctaon and that in arterial hypertension has een pointed out by Pickering (1955), who as never seen the malignant phase develop patients with coarctation. Granström 1951) has stressed the typical eye ground ndings in coarctation and the absence of assical hypertensive eye ground changes. The question of renal origin as cause for e hypertension in coarctation has been peatedly discussed (Rytand 1938; Steele, 941; Friedman, Selzer & Rosenblum, 1941; ealy, de Maria & Harris, 1950). Most uthors have determined the renal blood flow efore and after operation and found a somehat depressed renal blood flow before, increasing to normal afterwards (Genest, Newman, Kattus, Sinclair-Smith & Genecin, 1948; Bing, Handelsmann, Campbell, Griswold & Blalock, 1948; Harris, Sealy & de Maria, 1950). Some have taken this as evidence that a renal ischemia exists in coarctation, constituting a probable mechanism for blood pressure elevation. The idea has been rejected by others. However, even in hypertension due to experimental renal disease the renal blood flow does not have to be low, especially in the early stages of hypertension. It is thus hardly possible to arrive at any conclusion regarding the pathogenesis of hypertension from renal blood flow determination.

The comparison of the renal function in coarctation to the renal function in some congenital or acquired heart lesions may give some information. It is shown in the present study that the renal blood flow is equally low in coarctation, patent ductus arteriosus and auricular septal defect. After surgical correc-

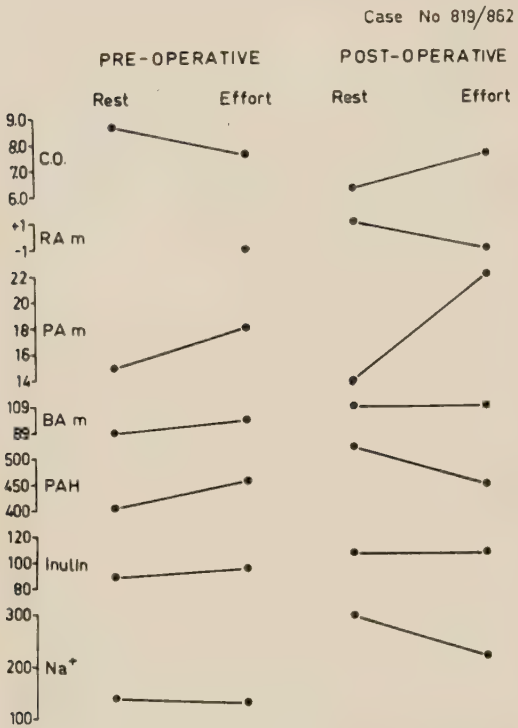


Fig. 1. Cardiac output (l/min.), renal clearances (ml/min.), sodium excretion μEq/min.) and blood pressures (mm Hg) during rest and after 15 min. exercise in two patients with coarctation of aorta before and after surgical correction of the stenosis.

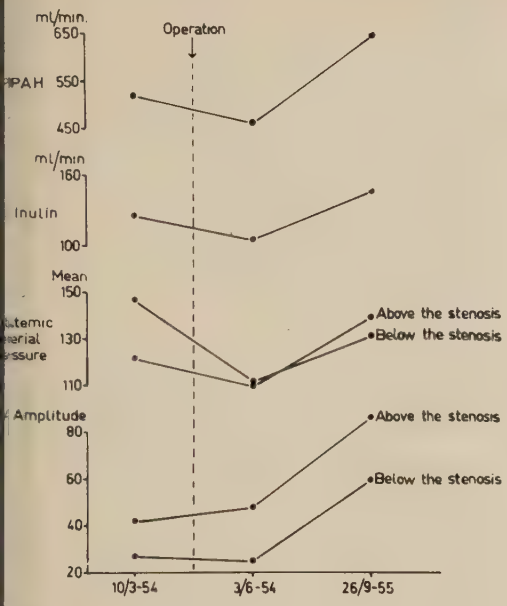
tion of the coarctation the renal blood flow increases as it does when these other congenital lesion are corrected. When the blood pressure and the renal blood flow were followed for a period after operation in a patient with hypertensive values the blood flow increased when the blood pressure rose. The renal blood flow thus rather followed the pressure in a mechanical fashion.

The patients who had some other heart lesions in addition to the coarctation had lower renal blood flow as a group than those with only coarctation. The renal blood flow was thus more closely correlated to the stat of the heart than to the degree of stenosi in the coarctation. This correlation was als better than any relation to the blood pressur above or below the stenosis, the blood pres sure fall in the stenosis or the pulse pressur in the aorta below the stenosis.

Once and a while patients may develo acute tubular necrosis following diodrast in jection in angiocardiography or aortography It is of especial importance to study the rena function in coarctation as all cases are re ferred to aortography. One of the patient in the present series developed acute tubula necerosis after aortography. He had anuri that lasted for seven days and recovere exretory function completely. Before the aortography the renal blood flow was normal He had, however, a complete coarctation The contrast was injected through the aort directly into the renal arteries. The kidney were thus under the influence of a heav dose of contrast medium. The developmen of anuria in this case was thus more due t the technique of contrast injection than t the renal function.

SUMMARY

The clearance of para-amino-hippurat and inulin was determined in 15 patient with coarctation of the aorta, in 8 cases simul taneous with determination of cardiac outpu and blood pressures and in 7 before and afte resection of the stenosis. In four patients th reaction to exercise was studied and in on the effect of a rapid intravenous infusion o dextran. The slight decrease of renal bloo flow found in the preoperative studies wa of the same magnitude as in patients wit atrial septal defect or patent ductus arterio



g. 2. Renal clearances and blood pressures in the rta above and below the stenosis before and 2 and 16 months after surgical correction of the stenosis.

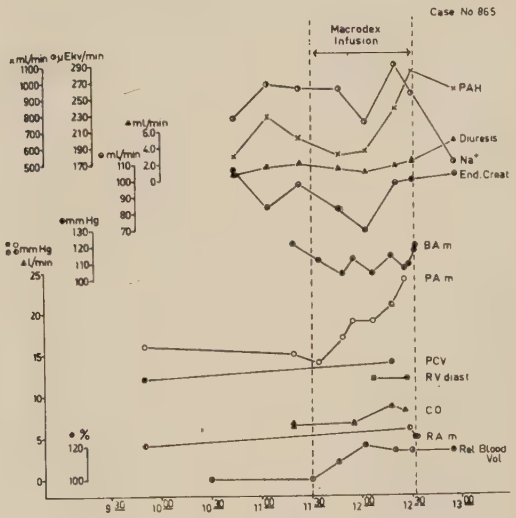


Fig. 3. Renal clearances, sadium excretion, cardiac output and blood pressures in a patient with coarctation of the aorta during a rapid infusion of dextran. The increase of para-amino-hippurate clearance corresponds to at least a 100 per cent increase in renal blood flow.

ss. The renal blood flow and sodium excretion reacted in a normal way to exercise and fusion of dextran and increased in some ses after operation. In one patient where e blood pressure was recorded below and ove the stenosis before, immediately after d 1½ years after operation together with nal clearances, the renal blood flow deeased when the blood pressure decreased d increased with increasing blood pressure. It is concluded that decreased renal blood w shows no relation to the origin of inased blood pressure in coarctation of the rta.

ACKNOWLEDGMENT

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Misses Ester Strandberg, Margaretha Söderling and Mrs. Anna Brita Olsson and Birgit Ekdal made the analyses of blood gases and clearance substances.

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EVALUATION OF THE p-TOLUENE-SULFONIC ACID METHOD FOR QUANTITATIVE DETERMINATION OF TOTAL CHOLESTEROL IN SERUM

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Although dating from 1885, the Liebermann-Burchard (1885) reaction for the determination of cholesterol is still in use as a serviceable method. The reaction is not specific for cholesterol. This observation was already made by the workers who devised the method, Liebermann & Burchard (1890). It was not developed into a quantitative method before 1910 by Grigaut (1910). Since the latter date a number of modifications of the reaction have been published, in which various details of the method have been studied, as follows:

1) reaction time
2) reaction temperature
3) effect of light
4) solvents
5) reagent mixtures and amounts, and
6) color for free and esterified cholesterol and the rapidity of color formation.

The differences between the various modifications of the method are actually very slight. Only the methods developed in recent years in which the extraction phase is eliminated show a more marked departure from the old theme. In this direct method of determination the use of a trivalent iron as oxidizer should be refrained from, as shown by the investigations of the norwegian workers Fürst & Lange (1954). Similar color tones obtained with various oxidizers were studied by Lifschütz in 1898 (1906). In the method developed by Pearson, Stern and McGavack (1953) in the United States the cholesterol is determined directly from the serum without the intervening phase of extraction and the color is developed with the aid of p-toluenesulfonic acid. This method is employed in the central laboratory of the Kivelä Hospital for the determination of total cholesterol. Some experiences in the use of this method are reported in the present paper.

PRINCIPLE OF THE METHOD

In the Liebermann-Burchard reaction a colored compound is formed by the action on cholesterol of concentrated sulfuric acid and acetic in a solution of acetic anhydride. The compound is of a green color and it was believed to be a halochromic sulfate of ketone. Later investigations have shown it to be cholesterilene, a sulfonic acid derivate, and barium salt $(C_{27}H_{43}O_3S)_2Ba$ has been prepared from it (Nath, Chakravorty & Chowdhury).

Reagents

1) Acetic anhydride,
2) p-toluenesulfonic acid solution: 10 g of p-toluenesulfonic acid dissolved in 100 ml of glacial acetic acid,
3) standard cholesterol solution: 200.0 mg of cholesterol dissolved in 100 ml of glacial acetic acid,

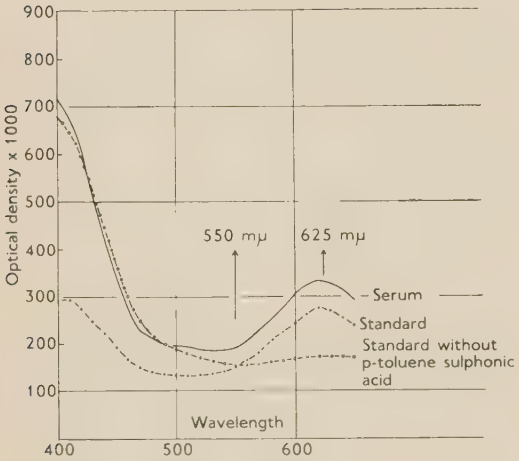

Fig. 1.

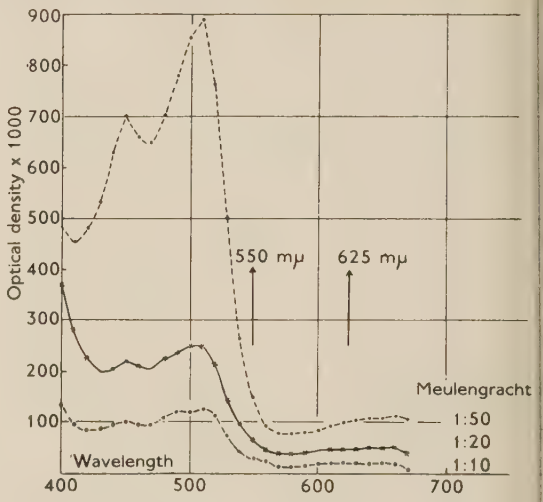

Fig. 2.

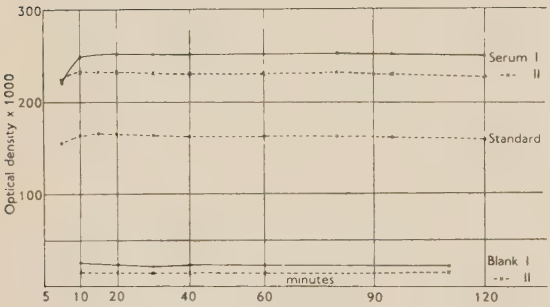

Fig. 3. 550 mμ.

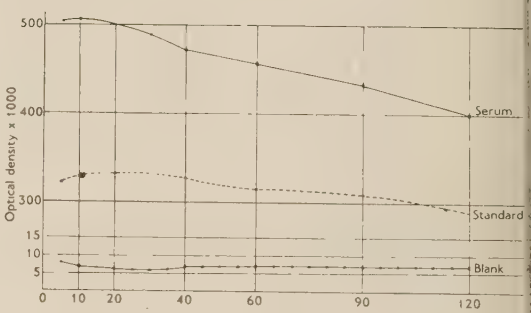

Fig. 4. 625 mμ.

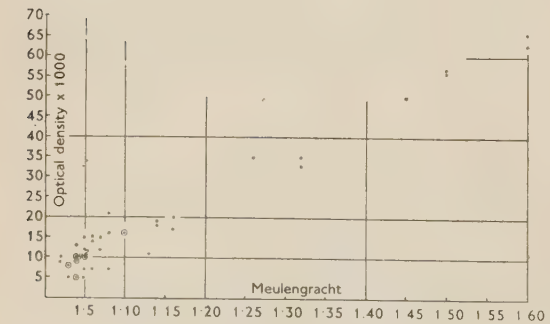

Fig. 5. 550 mμ.

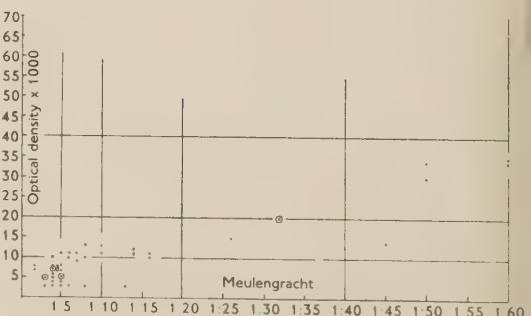

Fig. 6. 625 mμ.

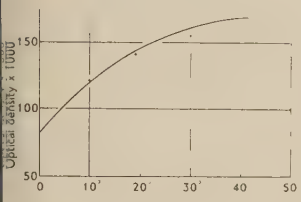

Fig. 7.

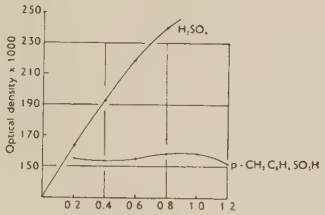

Fig. 8.

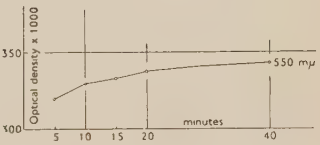

Fig. 9.

4) concentrated sulfuric acid, specific weight 1.84, and

5) glacial acetic acid.

All the reagents used are *pro analysis* products manufactured by E. Merck.

Procedure

The substances are pipetted into an ordinary test tube in the following order:

— 0.2 ml of serum,

— 0.2 » » glacial acetic acid.

— 1.0 » » p-toluenesulfonic acid solution,

— 3.0 » » acetic anhydride.

Mix thoroughly until all sediments are completely dissolved and let stand for 20 min. to bring the contents to room temperature. Then add 0.4 ml of ice-cold sulfuric acid and mix well once more. (Sulfuric acid is rather slowly miscible.) Keep at room temperature for 20 min. to again equalize the temperature. The measurement is carried out at a wave length of 550 mμ.

The standard solution is prepared in the same manner but substituting 0.2 ml of standard cholesterol solution for the acetic acid and 0.2 ml of distilled water for the serum.

A blank test is carried out in the same manner as the analysis, but for the serum is substituted 0.2 ml of distilled water. For the measurement of this solution the spectrophotometer is set at the zero point.

Another blank test is prepared for correction of the absorption due to interfering color substances in the serum. For this test 0.2 ml of serum and 4.6 ml of p-toluenesulfonic acid are used. This solution is measured against p-toluenensulfonic acid.

EXPERIMENTS WITH PURE CHOLESTEROL AND WITH CHOLESTEROL OF SERUM

Pearson *et al.* reported that the absorption curves for free cholesterol and cholesteryl ester run palallel, *i. e.*, the speed and intensity of color production are similar for both. The absorption curves for serum and the standard solution (with and without p-toluenesulfonic acid) are shown in Fig. 1.

The curves for the serum and the standard differ in the wave range 400—500 mμ, whereas they are parallel from about 520 mμ onward and reach maximum at 625 mμ. No absorption maximum is obtained for the standard solution prepared without p-toluenesulfonic acid. The absorption curves for the color of the serum in the blank test are seen in Fig. 2. The color intensity is stated in Meulengracht values. It is observed that these interfering colors drop abruptly just before the field of measurement is reached and that they are minimal at 580 mμ.

From these absorption curves it appears that the wave length of 625 mμ would be the most suitable point of measurement, whereas the extinction of the colors in Figs. 3 and 4 moves the point in favor of 550 mμ. At 550 mμ the values for the serum, standard solution and blank test have been found to remain constant for a relatively long time, *i. e.* for about 2 hours.

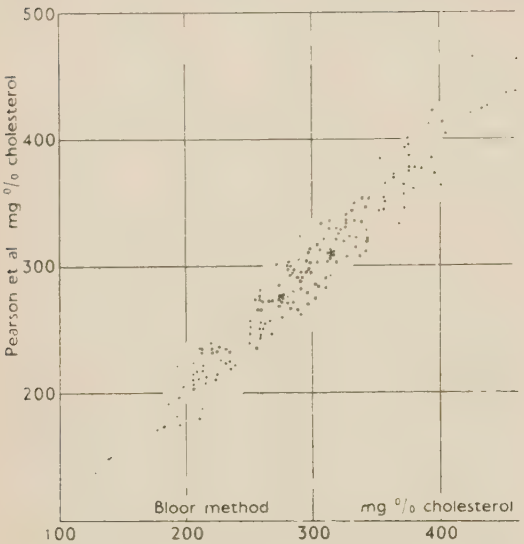

Fig. 10.

Fig.s 5 and 6 give values for interfering colors in the serum. These values were measured at wave lengths 550 mμ and 625 mμ as a functinon of Meulengracht values. No correction coefficient can be calculated for Meulengracht values and the color test should therefore be made separately from each sample. The effect of temperature on color development and the influence of minor inaccuracies in the measurement of the reagent were also studied and are shown in Figs. 7 and 8. The error due to changes in the temperature is practically eliminated since the standard test and the analysis are always carried out at the same temperature. Inaccurate pipetting of p-toluenesulfonic acid has no effect on the results, whereas that of sulfuric acid has a fairly great influence.

Fig. 9 shows the effect of an extension of the range of measurement after the addition of acetic anhyride. As will be observed, the situation remains fairly constant after 20 min.

Table I. *Cholesterol values in serum from several subjects.*

Subject	No. of determinations	Wavelength mμ	Mg/100 ml of cholesterol	
			Mean	Range
E. K.	10	550	206	204—210
A. K.	7	550	270	260—285
S. P.	10	550	302	297—310
P. 3.	6	550	341	338—350
P. 3.	6	625	343	337—355
A. I.	5	550	365	357—375
A. I.	5	625	364	360—369
J. 2.	5	550	423	415—440
J. 2.	5	625	425	412—447

Table I shows the results obtained in parallel tests and Table II the results of tests after the addition of pure cholesterol to the serum samples. The recovery tests appeared to have a negative trend. Table III shows the changes in the standard cholesterol values during different months.

Fig. 10 is a dotted graph showing a comparison of the new technique (Pearson, Stern & McGawac, 1953) with the old method (Bloor, 1922). It will be observed that the two methods have a high degree of correspondence.

DISCUSSION

The method as modified by Pearson (1953) takes into consideration the practical conditions in a routine laboratory. The readings are made from the maximum color intensity value and not at a closely specified time. The stability of the color is good and it is therefore suitable for serial tests. The reaction can be carried out at room temperature without the use of a water bath or of an exact temperature. Furthermore, the color is developed in light. The equipment may

Table II. *Percentage recovery of added cholesterol.*

ubject	No. of determinations	Wavelength mμ	Cholesterol		Cholesterol recovered	
			In serum mg/100 ml	Added mg	mg	in per cent
.I.	5	550	365	200	205	102.5
. I.	5	625	364	200	197	98.5
2.	5	550	423	200	202	101.0
2.	5	625	425	200	192	96.0
3.	6	550	341	200	189	94.5
3.	6	625	343	200	185	92.5
4.	5	550	153	100	93	93.0
4.	5	550	153	200	192	96.0
5.	5	550	190	100	88	88.0
5.	5	550	190	200	196	98.0

nsist of ordinary test tubes. The laborous xtraction phase, with loss of material, is iminated. The time of determination is us shorter, resulting in a considerable ving of time. The standard solution for ch day differs very slightly from the erage of 0.148 obtained for 100 days Table III).

The best point of measurement would be 25 mμ but this would require measurement fter a specified interval of time because of ss of color. The loss at wave lengths 560 nd 570 mμ is probably not too great for easurement with a considerable degree of ccuracy at also these wave lengths. An ncertain point in this method appears to be e measurement of the so-called color-blind alue. As was seen in Figs. 5 and 6, no uni-rm correction coefficient can be calculated r this value and the manner of reaction is so different. In our cholesterol determina-ons there was no explosive reaction with fferent p-toluenesulfonic acid products.

This method has been employed in the boratory of the Kivelä Hospital and has been found to be a rapid and accurate procedure which may be recommended for use as a routine method.

Table III. *Reproducibility of results of determinations of standard cholesterol solution during May 10—Sept. 21, 1955.*

Month	No. of determinations	Wavelength mμ	Optical density × 1000	
			Mean	Range
May	16	550	150	139—155
June	21	550	148	140—155
July	23	550	148	142—158
Aug.	24	550	147	139—155
Sept.	16	550	149	139—153

SUMMARY

An evaluation is made of a new method for the determination of cholesterol, which is well suited for routine analysis in hospital laboratories.

ACKNOWLEDGMENT

The author is indebted to Miss Valma Peltola for valuable technical assistance. Financial support was obtained from the Emil Aaltonen Foundation.

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Scandinav. J. Clin. & Lab. Investigation 207—212, 8, 1956.

THE EFFECT OF SALICYLIC ACID MEDICATION ON THE FORMATION OF ACUTE PHASE PROTEIN IN THE BLOOD

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The elevation of temperature as well as the production of acute phase protein (APP) are parts of the acute phase reaction. Other phenomena of this type are the increase of white blood cells, mainly due to the rise of polymorphonuclear leukocytes, and the shift to the left, the increase of blood sugar, etc. Previously we have shown that APP appears in the blood after experimentally induced fever in man (Hedlund, Frisk & Bucht, 1948). Simultaneously there is a change of the white blood-cell picture and of the electrophoretic pattern of the serum proteins, and the sedimentation rate rises.

In patients with acute rheumatic fever treated with large doses of salicylates, the APP vill disappear from the blood when the temperature decreases (Hedlund, 1947). Hence, it seemed of interest to study the influence of sodium salicylate medication specially on the production of APP, and on the changes in the sedimentation rate, the differential white count and the total leukocyte rate after induced fever.

MATERIAL AND METHODS

The experiments were carried out on six febrile convalescent patients showing a negative capsular swelling reaction and on one patient with fever and APP in his blood prior to the experiment.

Fever was induced by a suspension of formalin-killed *Aerobacter aerogenes*, the stock solution containing 100,000,000 bacteria per ml. A diluted solution of this vaccine as well as a solution of sodium salicylate were administered by intravenous drip. The drip rate was regulated according to the rise in temperature, a constant temperature of about 39° C being aimed at. Details regarding this technique hav been published (Frisk & Bucht, 1948). The drip apparatus had two containers which could be so connected that either one or the other, or both of them simultaneously, could be used. One container held vaccine and sodium salicylate (2 ml vaccine per 1000 ml 1 per cent (10 g/1000) sodium salicylate solution), the other 1000 ml 1 per cent sodium salicylate solution only.

Each patient received a total amount of from 1 to 2 ml vaccine. The amount varied depending on the fever reaction of the patient. Sodium salicylate solution, viz. about 100 ml per hour, was administered for fourteen hours, the total of sodium salicylate given to each patient varying between 11 and 14 g. Two patients (Figs. 7 and 8) were given smaller doses on account of toxic reactions.

Blood specimens for determination of APP, sedimentation rate leukocyte rate and differential white count were collected immediately prior to the drip being instituted and subsequently at three-hour intervals for 24 hours. After the first 24 hours the samples were taken daily. In the differential white count 200 cells were counted. The temperature was recorded every half or full hour (except in the cases referred to in Figs. 5 and 8, in which it was recorded of three-hour intervals) during the first 24 hours and subsequently every morning and afternoon.

The capsular swelling reaction of Löfström (1943) was used for the determination of APP in

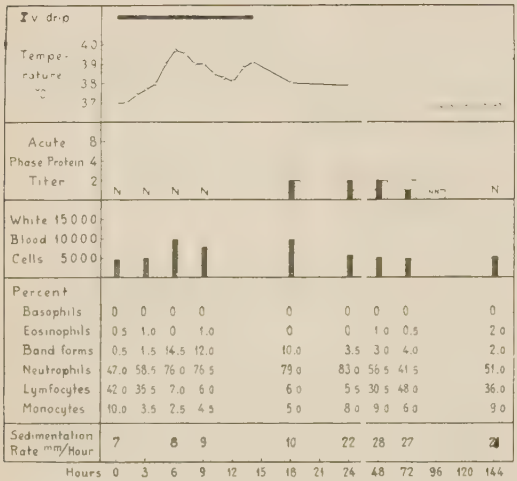

Fig. 1. Case 1. 2890/48. Male, 35 years of age.

Diagnosis: duodenal ulcer. Intravenous drip during fourteen hours. Total amount af vaccine 1.5 ml and of sodium salicylate 12.5 g.

N = negative APP reaction.
□ = pneumococcus type 23 B.
■ = » » 27.
▬ = » » 28.

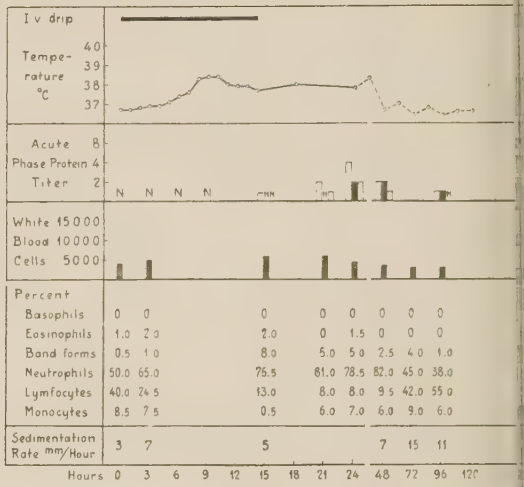

Fig. 2. Case 2. 3321/48. Male, 50 years of age.

Diagnosis: duodenal ulcer. Intravenous drip during fourteen hours. Total amount of vaccine 1 ml and of sodium salicylate 11 g.

N = negative APP reaction.
□ = pneumococcus type 23 B.
■ = » » 27.
▬ = » » 28.

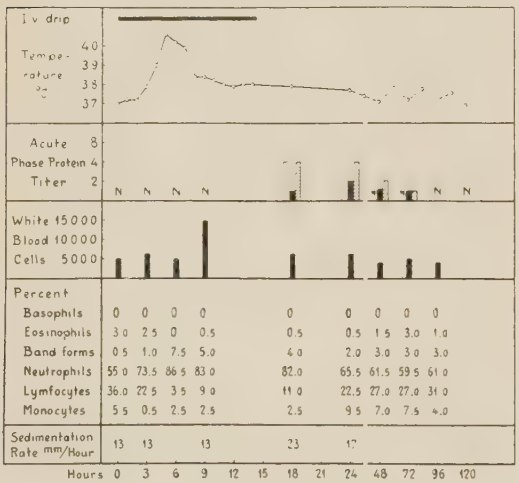

Fig. 3. Case 3. 2760/48. Male, 44 years of age.

Diagnosis: duodenal ulcer. Intravenous drip during fourteen hours. Total amount of vaccine 1.5 ml and of sodium salicylate 12 g.

N = negative APP reaction.
□ = pneumococcus type 23 B.
■ = » » 27.
▬ = » » 28.

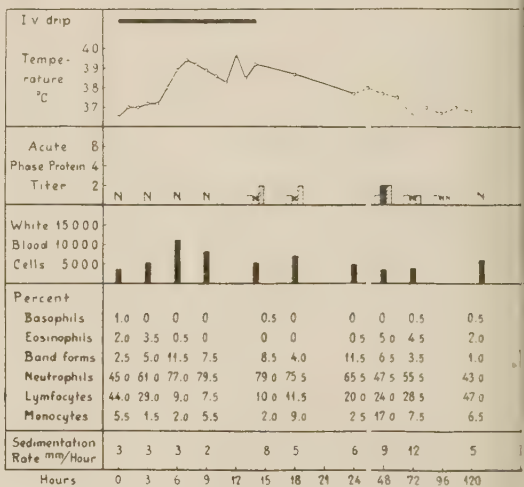

Fig. 4. Case 4. 2661/48. Male, 52 years of age.

Diagnosis: duodenal ulcer. Intravenous drip during fourteen hours. Total amount of vaccine 2 ml and of sodium salicylate 13 g.

N = negative APP reaction.
□ = pneumococcus type 23 B.
■ = » » 27.
▬ = » » 28.

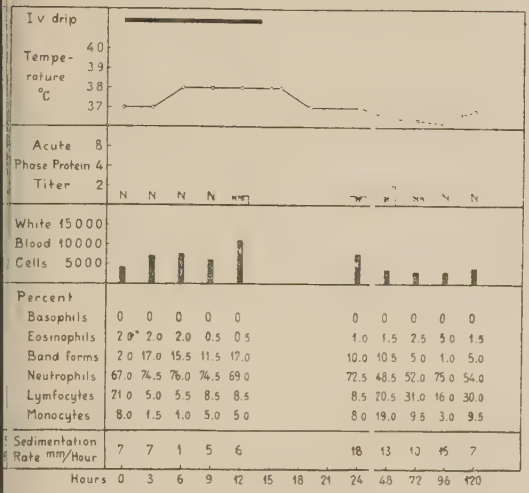

Fig. 5. Case 5. 3368/48. Female, 24 years of age.

Diagnosis: amenorrhea. Intravenous drip during fourteen hours. Total amount of vaccine 1.3 ml and of sodium salicylate 14 g.

N = negative APP reaction.
□ = pneumococcus type 23 B.
■ = » » 27.
▬ = » » 28.

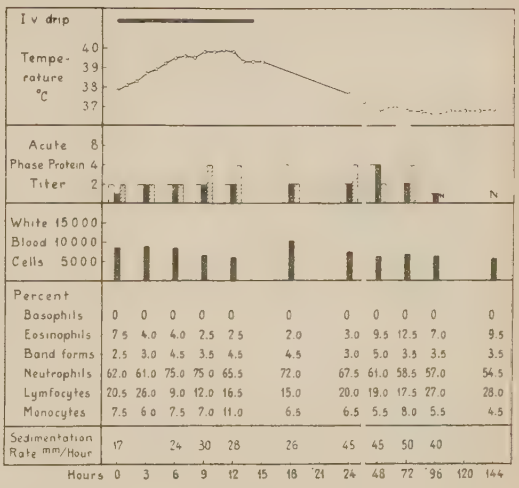

Fig. 6. Case 6. 2800/48. Male, 63 years of age.

Diagnosis: bronchial asthma. Intravenous drip during fourteen hours. Total amount of vaccine 1.8 ml and of sodium salicylate 11 g.

N = negative APP reaction.
□ = pneumococcus type 23 B.
■ = » » 27.
▬ = » » 28.

serum. In this investigation we have in addition to pneumococcus type 27, made use of type 11 A, 23 B and 28.

RESULTS

The results are presented in Figs. 1—8.

The temperature. — The temperature began to rise within three hours (except in case 5) after the drip was started. The fever began to decrease 2 to 4 hours after the drip was withdrawn, except in the cases 2, 7 and 8 in which a normal temperature was reached within a longer period.

The period required for attainment of a normal temperature varied within rather wide limits — for to fifty-eight hours (see Figs. 1—8). The temperature culminated 5 to 9 hours after the institution of vaccine treatment, except in case 8 where it was reached after the drip was withdrawn.

Acute phase protein. — APP in the blood could be demonstrated, at the earliest moment, 12—14 hours after the administration of vaccine (cases 2, 5, 7, 8). In two of these cases (5 and 8) the APP appeared after 12 hours, in spite of the fact that case 5 reacted with slight fever only. The titre maximum was reached 18—24 hours after the start of vaccine treatment in the other cases except in case 6 where the maximum was attained later. This case showed an APP reaction from the start. APP was demonstrated in the blood of cases 2, 4 and 7, 12 hours after the start of the temperature elevation, and after 9 hours in cases 5 and 8. The APP disappeared one to two days after the temperature had become normal.

The two cases demonstrated in Figs. 7 and 8 owing to comparatively well-marked

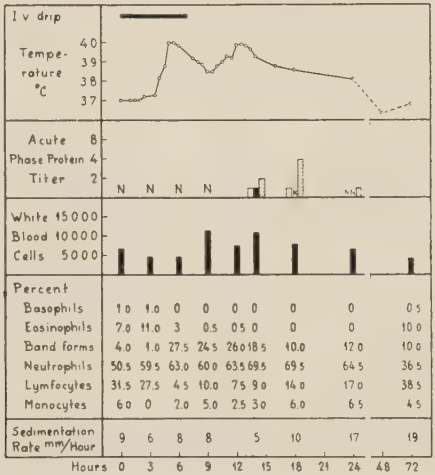

Fig. 7. Case 7. 3454/48. Female, 27 years of age.

Diagnosis: bronchial asthma. Intravenous drip during seven hours. Total amount of vaccine 1 ml and of sodium salicylate 7 g.
N = negative APP reaction.
□ = pneumococcus type 23 B.
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≡ = » » 28.

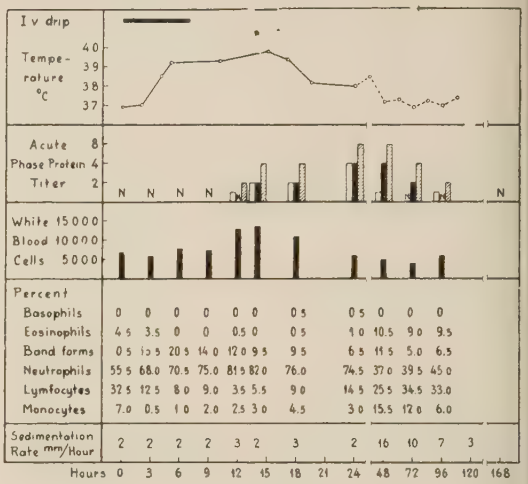

Fig. 8. Case 8. 3641/48. Male, 38 years of age.

Diagnosis: duodenal ulcer. Intravenous drip during seven hours. Total amount of vaccine 1.1 ml and of sodium salicylate 7 g.
N = negative APP reaction.
□ = pneumococcus type 23 B.
■ = » » 27.
≡ = » » 28.

toxic reactions received less vaccine and sodium salicylate than the other patients. None the less both of them showed typical APP. They received only 7 g sodium salicylate in 7 hours. Their reactions, however, followed the same course as those of the other patients who received 11—14 g in 14 hours.

No serum reacted with pneumococci 11 A. The capsular swelling reactions of the three types 23 B, 27 and 28 used on the whole ran parallel. Types 23 B and 28 were the most sensitive (Figs. 1—8) and they also gave the highest titres (1, 2, 3, 5, 6, 7 and 8). The importance of using a sensitive strain for the capsular swelling reaction is demonstrated in Fig. 5. In this case type 27 showed negative reactions during the whole experiment, while types 23 B and 28 yielded positive reactions.

The sedimentation rate. — The sedimentation rate culminated 1—3 days after institution of treatment. In five cases (2, 4, 5, 7, 8) it began to rise several hours after acute phase protein had appeared in the blood.

The white cell rate. — The curve of white blood cells on the whole parallelled the temperature curve. The increase of leukocytes could be demonstrated as soon as three hours after institution of the vaccine treatment. As long as the antigen was administered intravenously, there was an increase of the number of white blood cells, except in case 7 where a decrease was observed. The maximum of the elevation was reached within six to nine hours after institution of treatment.

The differential leukocyte count. — As will be seen from the differential leukocyte count, the increase of white blood cells depends upon a rise in the number of neutrophilic leukocytes and band forms of these. The lymphocytes, especially the small lymphoid leukocytes, were found to drop. The production of basophilic and eosinophilic leukocytes was not significantly altered.

DISCUSSION

Previous investigations have shown that subjects who receive typhoid vaccine and other bacterial proteins develop a transient leukocytosis with an increase in granulocytes, and a lymphopenia (Sundelin, 1939; Favorite & Morgan, 1946). *Inter al.* Ham & Curtis (1938) and Jager & Nickerson (1947) observed a rise in the plasma fibrinogen and in the sedimentation rate after injection of antigens. Jager & Nickerson showed that the antibody formation to the typhoid H and O antigens was suppressed in patients receiving salicylate medication. They also claimed to have shown that changes such as transient leukocytosis, lymphopenia, and rise in sedimentation rate following injection of a vaccine do not occur or are slight in extent. They held that high salicylate levels in the blood are necessary to obtain such an effect on the production of typhoid antibodies. They maintained levels between 300 and 400 mcg per ml plasma during a period of three weeks. Coburn (1943) showed in rheumatic subjects that the salicylate plasma content, by intravenous administration, of 10 grams of sodium salicylate daily, could be brought up to 330—550 mcg per ml Table IX, Coburn). Oral administration of 10 grams of sodium salicylate daily produced an average plasma level of 368 mcg.

In our experiments the plasma salicylate levels were not determined. However, it seems highly probable that the plasma salicylate level in our experiments were higher than 300 mcg as we administered as much as 11—14 g sodium salicylate during a comparatively short period — fourteen hours (cases 1—6). Evidence as to the effect of salicylate medication were the typical toxic symptoms, tinnitus, deafness, which all of our patients developed.

In a preceding study (Hedlund, Frisk & Bucht, 1948) the acute phase reaction was studied with the same method in patients receiving vaccine only. The results agree well with those obtained in the present investigation, in which vaccine and salicylate were given simultaneously. Sodium salicylate thus seemed to exert no influence on temperature, sedimentation rate, white blood count, or the production of APP.

SUMMARY

By administration of a vaccine, acute phase reactions were induced in human subjects. The effect of salicylic medication on this reaction was studied. The temperature, production of acute phase protein, sedimentation rate, differential leukocyte count and total white cell rate were recorded.

In these experiments sodium salicylate seemed to exert no influence on the temperature, sedimentation rate, white blood count and the production of acute phase protein.

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ISOLATION OF ACUTE PHASE PROTEIN BY MEANS OF CONTINUOUS ZONE ELECTROPHORESIS

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The properties of acute phase protein (C-reactive protein) have gradually become known. Its ability to react with pneumococcal C polysaccharide has been made the basis of methods of isolation and analysis (Tillett & Francis, 1930; Mac Leod & Avery, 1941; McCarty, 1947). More sensitive assays are founded on the nonspecific swelling reaction with pneumococci capsules (Löfström, 1943) and the ability of the substance to induce antibody formation in the rabbit (Mac Leod & Avery, 1941; Anderson & McCarty, 1950; McCarty, 1947). Important information about its properties as protein was also obtained in the fractionation studies by Abernethy & Avery (1941). Recently Wood, McCarty & Slater (1954) have improved the isolation technique and carried out accurate physico-chemical analyses. Starting from lipid-bound material, they have been able to prepare a crystalline protein in a good yield by a series of precipitation procedures. The acute phase protein thus obtained has been examined, *inter alia*, by electrophoresis and ultracentrifugation. The electrophoretic analyses have been carried out both in free solution and in a starch packing.

As a continuation of Löfström's work on acute phase protein, the occurrence of this substance has been studied from the clinical point of view by one of us (Hedlund, 1947). In the course of these investigations, which now comprise a vast case material, questions have often arisen concerning the chemical nature and biological function of the acute phase protein. The early physico-chemical studies of the substance, *e. g.* electrophoretic ones (Löfström, 1943; Perlman, Bullowa & Goodkind, 1943), did not give quite conclusive results. The uncertainties in the results could largely be ascribed to the difficulties of preparing pure material in quantities sufficient for analysis by current methods. Electrophoretic analyses, in free solution by the moving boundary method, were instead carried out on acute phase serum, but localisation of the active component relative to the normal ones was always difficult under the conditions used. Löfström (1943) used an apparatus described by Svensson (1942) with special arrangements for the separation of the different serum fractions. He showed that the nonspecific capuslar swelling substance belonged to the globulin fraction and moved faster than the γ-globulin. But he was unable to reveal the relationship of the active substance to the β- and α-globulins. Perlman *et al.* (1943) found that the C protein migrated with the α-globulin fraction. In consequence of his promising fractionation experiments in sand

columns. Svensson suggested in 1944 that acute phase serum should rather be studied electrophoretically by this technique. After a continuous-working apparatus for electrophoresis in powder columns had been developed (Svensson & Brattsten, 1949; Brattsten, 1952, 1955), such fractionations of acute phase serum have been carried out (Hedlund & Brattsten, 1955). This paper reports the fractionation of 5 human sera containing acute phase protein. The material was obtained from some typical cases of acute infections. The observed migration rate of the active component was in the same range as that observed by Wood and co-workers (1954). Under comparable fractionation conditions these workers found the active substancee in the γ-globulin group.

The substance has been assayed by means of Löfström's capsular swelling technique. For comparison of the methods of analysis, each sample containing the active substance has also been tested with the aid of C-reactive protein antiserum (CRPA).

MATERIAL AND METHODS

Blood was taken from five patients treated for acute infections in the Hospital for Infectious Diseases, Stockholm. The blood was stored overnight in a refrigerator. Serum was then isolated by centrifugation and kept frosen at —20° C until used in the fractionation. The diagnoses according to case histories were as follows:

Preparation I: 4010/54 B. D.: Angina tonsillaris streptococcica.
4233/54 H. D.: Sinusitis maxillaris acuta and Myalgia nuchae.

Preparation II: 3243/55 J. D: Enteritis acuta.

Praparation III: 4247/55 E. D: Bronchopneumonia dx + Sinusitis maxillaris acuta + Syndroma Stevens-Johnson + Diabetes mellitus.

Preparation IV: 4218/55 J. D: Pneumonia ac.

Electrophoretic fractionation

Each lot of serum, 15—20 ml, was diluted to a protein concentration of 1—2.5 per cent and dialysed at 4° C. The resulting solutions were fractionated by continuous zone electrophoresis as described by Brattsten (1952, 1955). The fractionation cell contained a starch packing as support for the electrolyte solutions. The migration was conducted in a medium of constant composition at pH 8.5. Either veronal buffer of ionic strength 0.03 or veronal-acetate buffer of ionic strength 0.06 was used. The serum solution was injected at a rate of 3—4 ml per hour into a section of 1 cm^2 of the top area. The fractions were collected at the bottom plane of the cell. The distance between each two adjacent outlet points was only 0.5 cm. The bath current, regulated by means of a two-stage transductor amplifier, followed the minor fluctuations of the liquid flow rate. It was in the range of 250 mA when buffer of ionic strength 0.03 was used, and 340 mA at ionic strength 0.06. The cell temperature was read with a thermocouple. It amounted to about 15° C in the middle of the packing.

The total concentration of protein in the fractions was determined by measurements of the UV absorption at 284 mμ. A Beckman spectrophotometer, Model DU, was used.

Electrophoretic analysis.

A couple of the acute phase sera were analysed by moving boundary electrophoresis in free solution. In these experiments a Tiselius-Svensson electrophoresis apparatus of type LKB 3021 was used. The electrolyte medium consisted of veronal buffer of ionic strength 0.1 and pH 8.6.[1]

Electrophoresis in filter paper (Whatman No. 1) was carried out on samples of the enriched fractions containing acute phase protein (see below). The electric migration of the active fractions was correlated to that of the ordinary serum components by application of reference spots of whole serum

[1] The authors are indebted to Mr. Gösta Forsling, Institute of Biochemistry, Uppsala, for carrying out the analyses by the moving boundary method.

in the paper strips. The technique of Wieland & Fischer (1948) was used. The paper strips were stained with Amidoschwarz 10 B Bayer as described by Grassmann, Hannig & Knedel (1951).

Activity analysis

By the fractionation procedure each component in the original serum suffered a total dilution of about 6—7 times. Samples for the activity tests were prepared by removing the veronal ion, adding small amounts of calcium, and increasing the protein concentration. Thus, after dialysis against Ringer's solution, the fractions were enriched by evaporation in cellophane bags until each component was present in a concentration approximately equal to or higher than that in whole serum. The ionic strength was regulated by repeated dialysis against Ringer's solution.

The acute phase protein was determined both by the capsular swelling reaction as described by Löfström (1943) and by precipitation with the commercially available C-reactive protein antiserum (CRPA) (Schieffelin and Company, New York). For the capsular swelling reaction we have, in addition to pneumococcus type 27, also made use of type 23 B. In the routine procedure higher titers than 32 are very seldom recorded. In this case, however, we have, by dilution of the fractions, been able to record titers up to 128. The directions given by the manufacturer of CRPA have been followed. Thus the strength of reaction has been recorded from 0 to 6+.

The samples have been examined immediately after the electrophoretic experiments. They have been stored in the frozen state at — 20° C or, for shorter periods, at + 4° C (*e. g.* overnight). There has been no change of activity when the samples have been reexamined after periods of storage of 4—10 months.

EXPERIMENTAL RESULTS

A preliminary fractionation experiment was carried out in order to localise the acute phase protein in relation to the ordinary electrophoretic components. Hence the maximum available resolution was used in this case. The protein components were

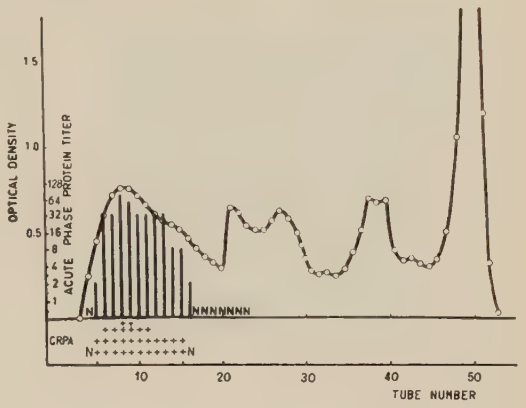
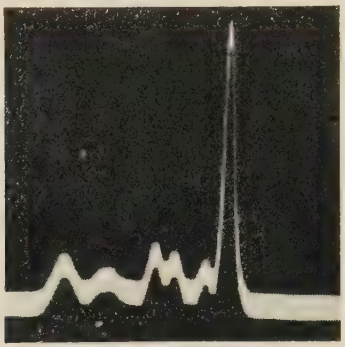


Fig. 1. Fractionation of a mixed sample of acute phase serum from two patients with Streptococcal pharyngitis and Maxillary sinusitis, respectively. The acute phase protein titer of each serum was 16 according to the capsular swelling reaction. Concentration of injected protein solution: 1.5 per cent. Buffer medium: veronal-sodium veronal, ionic strength 0.03, pH 8.5. Rate of fractionation: 0.8 ml of undiluted serum per hour. Electric current: 245 mA. Cell temperature: 17° C.

a. Distribution of total protein and acute phase protein in the fractions. The total protein concentration is represented by the optical density at 284 mμ (full curve) and the acute phase protein titer according to the capsular swelling reaction by the bars. "N" indicates negative reaction in the fractions. Pneumococci suspension: type 27. The titration results with CRPA are given below the diagram.

spread across a total distance of 25 cm when they left the cell. The resulting protein distribution, as recorded by the optical density measurements, is shown in Fig. 1 a. The experimental data have been collected in the legend to the figure.

The approximate site of the activity was first determined on samples, each of which contained material from one of the main electrophoretic groups. Thus eight pools were prepared from the fractions 4—13, 14—19, 20—23, 24—31, 32—35, 36— 41,



b. Analysis of whole serum by moving boundary electrophoresis. Optical pattern after the passage of 156 amp.secs., ascending boundaries.

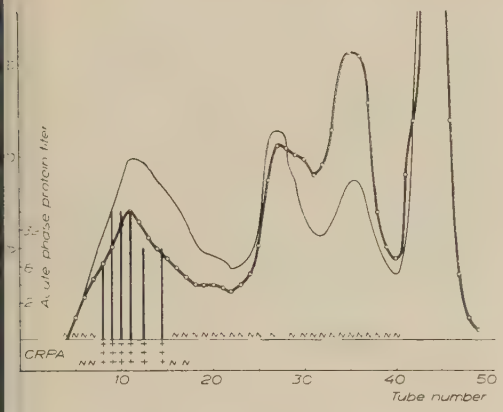
43—47, 48, and 53. Ten ml of each fraction entered into the respective pools. The material was then concentrated as desribed above. All the resulting solutions were free from active substance except that containing the fractions 4—13, the titer of which was as high as 32. This result showed that the reactive substance resided in the γ-globulin fraction of the sera investigated. On the basis of this result each 15 ml of the fractions 4—23 were analysed separately. The titration values are presented in Fig. 1 a. Acute phase protein was demonstrated in fractions 5—16. The highest titers, 32—96, were found in the fractions 6—13, which correspond to the maximum of the γ-globulin distribution curve. The precipitin test using the CRP antiserum showed C-reactive protein in fractions 5—15. The highest titers were found in 6—11, with a maximum, or 4 + reaction, in fractions 8 and 9. The validity of the fractionation results was checked by anaytical electrophoresis experiments in free solution and in filter paper. The fractionation pattern, Fig. 1 a, was compared with the refractive index gradient pattern

c. Electrophoresis in filter paper of samples of fraction no. 9 and whole serum. Ionic strength of buffer: 0.08.

from a moving boundary experiment (see for example Fig. 1 b). The similarity betwen the two diagrams is apparent. The observations with regard to the mobility of the active substance were confirmed by electrophoresis experiments in filter paper. They showed that the fractions containing the acute phase protein migrated as γ-globulins exclusively (see Fig. 1 c).

In the subsequent fractionations advantage was taken of the ease of isolating the γ-globulins from all serum components of greater mobility. Thus, the rate of fractionation was increased by the use of a higher protein concentration in the injected solution, and by enhancement of the ratio between the liquid flow and electric current.

Fig. 2 shows the result of a fractionation carried out in the same buffer as that initially



g. 2. Fractionation of acute phase serum from a se of fever of unknown cause. Distribution of al protein and acute phase protein. Symbols as in g 1 a. Titer of serum: 16. Protein concentration injected solution: 2.4 per cent. The fractiona- n was carried out in veronal buffer of ionic ength 0.03 and pH 8.5. Rate of fractionation: ml of undiluted serum per hour. Electric cur- t: 228 mA. Cell temperature: 14° C. Acute ase protein titer against Pneumococci suspensions type 27 and against CRPA. — For comparison, fractionation pattern of a normal serum has en included in the diagram (thin line). The frac- nation conditions were the same in both cases.

ed but at a lower electrophoretic resolution. ne rate of separation was raised to 1.6 ml undiluted serum per hour, *i. e.* by a factor 2. The activity tests showed, as before, e presence of the acute phase protein in the globulin portion of the serum proteins. ractionation of a normal serum immedi- ely before and after the application of that ntaining acute phase protein gave a pattern hich is also shown in Fig. 2. Since the lution of the serum and all other experi- ental conditions were the same, the two tterns are directly comparable. Parti- larly noticeable is the abnormally low con- ntration of γ-globulin in the acute phase rum.

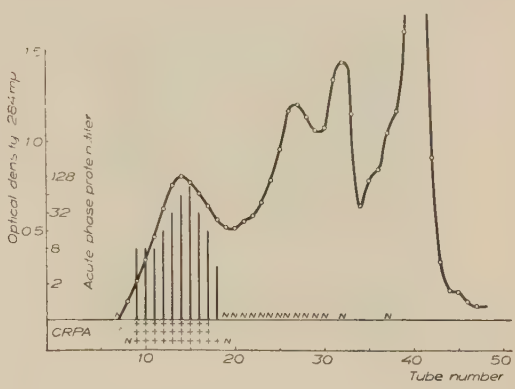
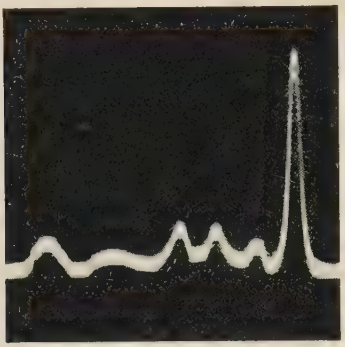


Fig. 3. Fractionation of acute phase serum from a case of pneumonia. Titer of serum: 16. Protein concentration of injected serum solution: 2.4 per cent. Fractionation carried out in veronal-acetate buffer of ionic strength 0.06 and pH 8.5. Rate of fractionation: 1.3 ml of undiluted serum per hour. Electric current: 337 mA. Cell temperature: 17° C.

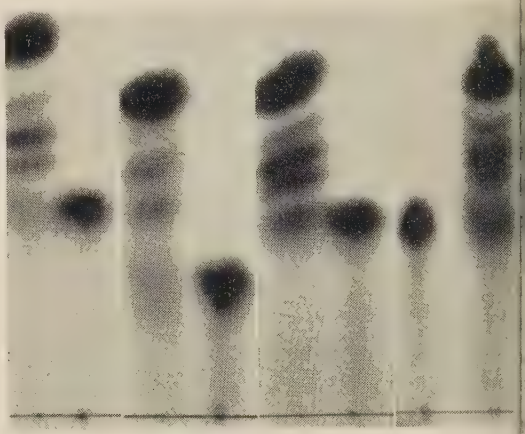
a. Distribution of total protein and acute phase protein in the fractions. Symbols as in Fig. 1 a. The acute phase protein titers listed are against Pneumococci suspensions of type 27 and CRPA.

In two further fractionations, the ionic strength of the medium was increased to 0.06 by the addition of sodium acetate to the veronal buffer. The distribution curves obtained in these experiments are shown in Figs. 3 a and 4. The titration results are in qualitative agreement with the previous ones. The optical pattern from an analysis by moving boundary electrophoresis of one of the fractionated sera is shown in Fig. 3 b.

In the cases of the two last-mentioned fractionations, the active fractions were examined by paper electrophoresis at ionic strengths varying between 0.04 and 0.1. These changes of the migration conditions had no apparent influence on the migration



b. Analysis of whole serum by moving boundary electrophoresis. Optical pattern after the passage of 215 amp.secs., ascending boundaries.



c. Electrophoresis in filter paper of samples of fractions No. 15 and whole serum. Ionic strength of medium in the respective experiments: 0.04, 0.05, 0.08, and 0.1.

of the active substance relative to that of the ordinary serum components, as illustrated by Fig. 3 c.

DISCUSSION

The fractionation experiments reported here are in general agreement with the findings of Wood, McCarty and Slater (1954) with regard to the mobility of the acute phase protein relative to the ordinary electrophoretic components in serum.

However, there still remains a certain variation in the observations of the mobility. By electrophoresis in free solution Wood and coworkers found a mobility which coincided with that of the β-globulin group, but in their starch column experiments the migration was slower and comparable with that of a fast γ-globulin. All our experiments have shown a still lower migration rate of the acute phase protein. As evident from Figs. 1—4 the distribution of this substance in the fractions approximately coincided with that of the major γ-globulin component.

These small discrepancies are difficult to explain conclusively at the present. The material studied has come from different sources and different experimental techniques have been employed. Accurate mobility values can hardly be derived from the experiments in starch packings. Relative determinations — expressed in reference to suitable substances of known mobility — are generally sufficient for the purposes of preparative electrophoresis, but with regard to the characterisation of the acute phase protein it must be remembered that not only the behaviour of this substance itself but also the mobility variation of the ordinary electrophoretic components of serum are reflected in the measurements.

When electrophoresis is carried out in the presence of a supporting capillary system, sorption to the solid phase may interfere with the electric fractionation process. In batch experiments noticeable sorption necessarily causes a reduced migration rate. In the continuous-working process, the steady-state

nigration is largely unaffected by reversible orption, since saturation takes place in the aths of the zones during the initial period f the transfer. Therefore, if experiments ith the two techniques do not indicate the ame mobility under conditions equivalent n other respects, this may be explained as result of sorption to the solid phase. The iscrepancy between the findings of Wood nd coworkers and our results can hardly e interpreted as due to sorption, however. f the acute phase protein were more rongly adsorbed than the other serum roteins, one would not expect to find the mallest migration rate under the conditions f continuous fractionation. Nor was it pos- ble to establish any reduction of the activity f the fractions during the initial periods of he experiments. An assumption of less finity to starch of the active substance than f the remaining proteins is not consistent ith a greater rate of migration in free olution than in the starch packing, as und by Wood and coworkers.

Wood and coworkers present experimental vidence that neither the differences in com- osition of the electrolyte media used, nor irect interaction of the acute phase protein ith the other serum proteins gave rise to ae observed shift of the relative mobility. hese experiments were carried out at a mparatively great ionic strength, appar- ntly 0.3 and 0.1, and pH 7.3—8.0. The xperiments reported herein indicate that the obility of the acute phase protein relative the γ-globulin is equally independent of the nic strength in the range of 0.03—0.1. The reparative fractionations were carried out ionic strength 0.03 or 0.06. In the paper ectrophoresis experiments the ionic strength

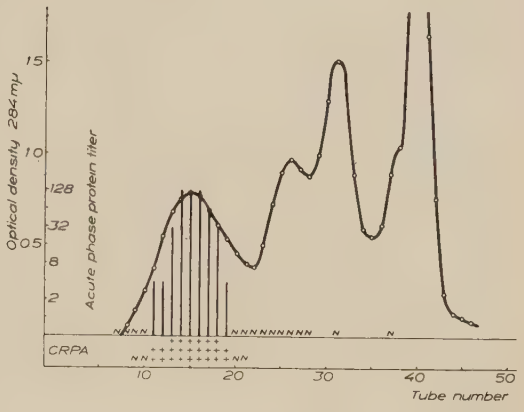


Fig. 4. Fractionation of acute phase serum from a case of pneumonia. The distribution of total protein and acute phase protein in the fractions. Symbols as in Fig. 1 a. Titer of serum: 16. Protein concentration of injected serum solution: 2.4 per cent. Fractionation carried out in veronal acetate buffer of ionic strength 0.06 and pH 8.5. Rate of fractionation: 1.3 ml of undiluted serum per hour. Electric current: 340 mA. Cell temperature: 17° C.

was further increased to 0.1. The latter experiments were, in the first place, meant as a general check on the fractionation conditions in the preparative apparatus. However, the samples selected were highly concentrated and contained, according to the analyses, a maximum amount of acute phase protein. Under these conditions it could be expected that a marked dependence of the migration rates on the ionic strength in the new media would cause a perceptible spreading of the spots in the paper. Such an effect has not been noticed.

The observed discrepancies may rather be related to the pH differences in the experiments. The isoelectric point of the γ-globulin is about 7.3 (Alberty, 1949), whereas the acute phase protein is isoelectric at pH 4.8 (Wood *et al.*, 1954). If, therefore,

the two proteins have the same mobility at pH 8.6, as found by us, the more acidic acute phase protein can be expected to move faster than the γ-globulin at a pH of 7.5—8.0.

The distribution of the acute phase protein in the fractions, according to Figs. 1—4 shows a good qualitative correlation to the optical density curve representing total protein. Since previous analyses on normal serum have shown that the sequence of fractions contains substances of successively changing mobility (Brattsten, 1955), the acute phase protein is apparently electrophoretically heterogeneous in these experiments. The titration values are, however, recorded on a logarithmic scale, and the drop in activity on both sides of the apex is much more pronounced than the decrease of total protein. The distribution found suggests that the acute phase protein is appreciably more homogeneous than the γ-globulin component of serum.

The site of the acute phase protein in the fractions of least mobility is very advantageous from the preparative point of view. Firstly, by displacing the faster components outside the γ-globulins zone it is possible to remove as much as 85—90 per cent of the total protein from the active substance. The latter figure is valid for the fractionation depicted in Fig. 2. The pattern shows that the proportion of γ-globulin in the serum was in this case unusually small, yet the activity of the fractions was in the maximum range. In all the fractionations, Fig. 1 a, 2, 3 a, and 4, the α-globulin components were present in abnormally increased concentrations (cf. Longsworth, 1939; Wiedemann, 1946). The electrophoretic purification would hardly have been equally efficient, had the component under investigation migrated in this range instead of with the γ-gobulin. In addition, the jump in mobility between the γ globulin and the next faster main component is considerably greater than that between any of the other consecutive members of the electrophoretic spectrum. It follows that a minimum amount of electricity is needed for the separation in the present case. Secondly, Abernethy & Avery (1941) showed that the acute phase protein does not precipitate from a serum solution when ammonium sulfate is added to half saturation of this salt. Accordingly, in the fractions which contained only γ-globulin and acute phase protein the activity of the liquid phase did not change upon precipitation of all the γ-globulin present by addition of ammonium sulfate. This behavior has been established experimentally. For these reasons it seems that electrophoretic fractionation, carried out under suitable conditions and followed by removal of contaminating γ-globulin by salt precipitation, affords a convenient method for preparing acute phase protein. Since this procedure does not involve any precipitation of the active substance, it can probably be regarded as a mild treatment of the protein material. The active substance derived from the acute phase sera according to the outlined procedure will be investigated further.

The four fractionation experiments also show that the medium of low ionic strength, 0.03, gave as clear a separation as the more concentrated one. The excellent resolving power in the former case contributes greatly to the efficiency of the process, and apparently there are no reasons why a higher salt content should be used. The experiments

indicate that a further increase of the fractionation rate can be obtained by the introduction of broader protein zones and a higher total protein concentration.

As mentioned already, the determination of the acute phase protein was based on three independent sets of measurements, namely, the non-specific capsular swelling reaction against pneumococci types 23 B and 27 and precipitation by C-reactive protein antiserum. The agreement was excellent. Type 27 was more sensitive than typ 23 B. Capsular swelling (titer 2) without a positive CRPA reaction was only found in fraction No. 16 (Fig. 1 a).

The capsular swelling method in these experiments was superior to the CRPA method as it was possible to determine the maximum of the acute phase protein content in the fractions. In the experiment seen in Fig. 1 a, there was good agreement between the two methods, in as much as both the capsular swelling and CRPA titers showed a maximum in fractions 8 and 9. In the other experiments, however, it was only possible to determine the titer maximum with the capsular swelling reaction, while none of the precipitates given by CRPA was higher than 3 mm, corresponding to a 3 + reaction.

SUMMARY

An examination by continuous zone electrophoresis at pH 8.5 of some acute phase sera from patients suffering from acute infections has been performed.

The sera and the fractions were tested for acute phase protein (C-reactive protein) by the capsular swelling reaction described by Löfström and by precipitation with the commercially available C-reactive protein antiserum (CRPA).

It was shown that the acute phase protein in human serum migrated with the γ-globulins.

There was a good correlation between the titers of C-reactive protein determined by the two methods used, the capsular swelling reaction and CRPA test.

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QUANTITATIVE DETERMINATION OF FRUCTOSE BY MEANS OF A THYMOL-HYDROCHLORIC ACID REAGENT

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Many methods for the determination of fructose in urine and other biological systems have been described (see Roe, 1934). However, these methods are not entirely specific for fructose particularly in the presence of large amounts of glucose. A notable advance was made by Roe (1934) who adapted Seliwanoff's reaction to permit the determination of fructose in the presence of glucose in concentrations up to 3 mg/ml. If fructose had to be determined in the presence of larger amounts of glucose it was necessary to use correction curves. Moreover the color formed is not completely stable as pointed out by Pogell (1954). Dische & Borenfreund (1951) have described a method for the determination of ketosugars. The reagents they used are sulfuric acid cystein and carbazol. In their method the development of the color for fructose takes 18 hours, the optical density for gluose being one per cent of that for fructose.

In the present investigation a mehod is described which allows the determination of fructose in amounts as low as 25 μg/ml. Glucose and other common aldosugars in amounts up to 5 mg/ml do not seriously interfere with the fructose determination. On the other hand sorbose gives almost the same color as fructose.

Sucrose and inulin have color equivalents corresponding to their fructose contents and the color develops without hydrolysis prior to the determination. The method is based on the red color formed when fructose is heated with tymol in the presence of hydrochloric acid, a reaction first described by Baldeon (1949). The method has been applied to the determination of fructose in urine and blood. By the use of a preliminary treatment with charcoal to remove extraneous chromogens it is possible to determine fructose in concentrations as low as 0.5 mg/ml in urine even in diabetics with a glucose concentration of 50 mg/ml. In blood the lower limit for the determination of fructose is about 0.5 mg/ml. The method is also directly applicable to the determination of ketoses in alcoholic eluates from chromatographic colums.

Recently three other new methods for the determination of fructose have been described, although these have not been directly applicable for our purposes.

Pogell (1954) used the color formed when fructose and skatole are heated with hydro-

chloric acid. The highest amount of glucose which did not cause any serious disturbance was five times that of fructose. In addition some color instability and high blank values were noted.

Bonting (1954) used a modified anthrone reaction for the determination of fructose and glucose on an ultra micro scale. The method is based on an observation that fructose but not glucose gives color at room temperature with the anthrone reagent. For the development of color glucose heating was necessary.

Cole, Hanes, Jackson & Laughman (cited by Bell, 1955) have modified the original method of Roe. It is claimed that the color formed by ketoses is 100—200 times more intense than the color formed by the corresponding aldoses.

DETERMINATION OF FRUCTOSE IN ALCOHOLIC SOLUTIONS

METHOD

Reagents

1. Stock solutions containing 10 mg per ml of fructose in 0.25 per cent benzoic acid solution. This solution remains stable in the refrigerator for at least one month.
2. Standard solutions containing between 250 and 4000 μg per ml are prepared from the stock solutions by dilution with water. For the analysis these were further diluted 1:10 with 96 per cent ethanol to give a final ethanol concentration of 86 per cent.
3. 1 per cent thymol in 9 per cent alcohol.
4. Concentrated hydrochloric acid.

Procedure

The reagent is prepared by mixing equal volumes of thymol solution and hydrochloric acid. This mixture is stable for one day.

One ml of the solution to be analysed, containing 25—400 μg of fructose dissolved in 86 per cent alcohol is pipetted into a thin-walled 15 × 120 mm test tube and 3 ml of the thymol-hydrochlorich acid reagent is added. After careful mixing of the contents the test tubes are placed in a water-bath and kept at 70° C for twenty minutes. They are then chilled in an ice water bath for twenty minutes. The color is stable for at least thirty minutes thereafter. The color intensity is measured at 505 mμ by the usee of 1 cm cuvettes in a Beckman spectrophotometer, model B, or similar apparatus. The test tubes are kept in the ice water bath until immediately before reading.

Calculation

A series of standards containing at least four different known concentrations of pure fructose and two blanks are treated in the same way as the samples to be analysed. The blanks consist of 1 ml of 8 per cent alcohol treated in the same way as the samples.

The readings of the standards are plotted against the known concentrations and a calibration curve is drawn from which fructose concentrations in the unknown samples may be read of directly. Since the standard curve is practically identical from one time on another, the standards can be omitted when accuracy is not essential.

RESULTS AND DISCUSSION

Absorption spectrum. The light absorption of the colored reaction product at wavelengths between 380 and 620 mμ is shown in Fig 1. The maximum absorption is at 505 mμ.

A typical standard curve is plotted in Fig. 2 showing that the relation between color intensity and fructose concentration is linear up to 400 μg per ml. Up to 800 μg the relation was not completely linear, a small decrease in the color yield being noted.

In Table I the recovery of different amounts of fructose is shown.

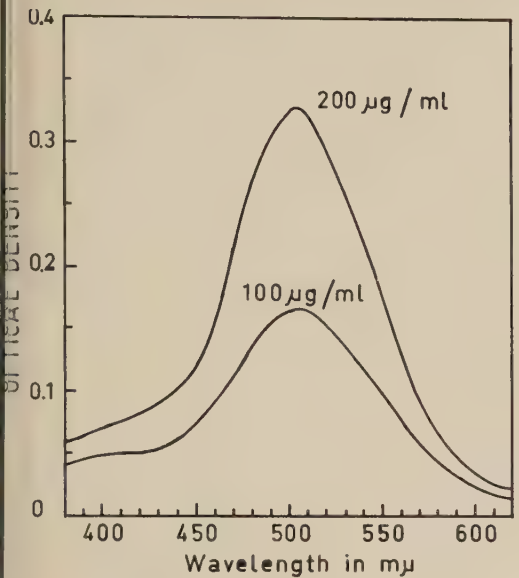

Fig. 1. Absorption spectrum for fructose in the thymol-hydrochloric reaction.

Specificity. It was observed that the reaction is almost specific for ketosugars, sorbose giving almost the same color yield as fructose. The reagent also gives color directly with sucrose and inulin *i. e.* without hydrolysis prior to the determination. It thus seems possible to use the thymol reagent for the determination of inulin in connection with investigations of renal inulin clearance.

However, this point has not been further investigated. The aldohexoses and aldopentoses gave very low color values as shown in Table II. The effect of glucose is treated in detail below.

Influence of glucose. One of the main difficulties in the determination of low levels of fructose in biological fluids is the interference of glucose. The recovery of fructose from solutions containing different amounts of glucose is seen in Table III.

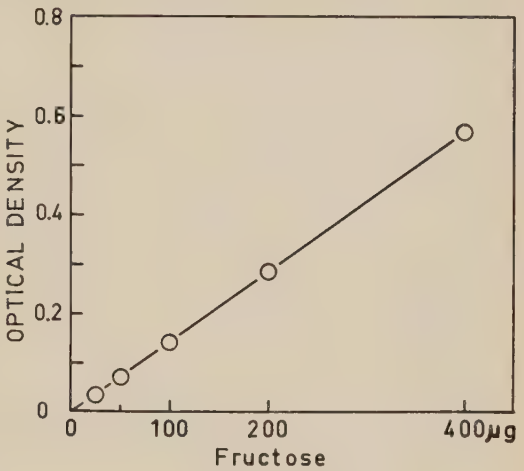

Fig. 2. Standard curve for fructose dissolved in 90 per cent alcohol and treated as described in the text.

Solvents. The colored reaction product formed is soluble in alcohol. If the alcohol concentration in the sample is less than 70 per cent the product begins to precipitate. It was also observed that the amount of the color formed from a given amount of fructose increased with increasing alcohol concentration. On the other hand the low solubility of monosaccharides in absolute ethanol is well known. A concentration of 85—90 per cent ethanol in the sample seemed to give optimal conditions.

The effect of temperature and time of heating are shown in Figs. 3 and 4 respectively.

From the figures it is evident that increased temperature causes an increase in color formation. At the same time however, the false response of glucose in the fructose determination becomes relatively more pronounced.

The same applies to prolonged heating time. The conditions selected for the analysis

Table I. *Recovery of different amounts of fructose.*

Fructose Added μg	Fructose Found μg	Recovery in per cent
25	25	100
50	48	95
100	94	94
200	198	99
400	198	99
400	416	104

Table III. *Recovery of fructose from solutions containing different amounts of glucose.*

Fructose in μg	Glucose in μg	Fructose recovered in μg	Recovery in per cent
100	3000	101	101
100	3000	101	101
100	5000	103	103
100	10000	117	117

represented a compromise among these factors.

Thymol concentration. The double amount of thymol, *i. e.* a 2 per cent tymol solution, has an inappreciable effect on the color formation. Also the thymol concentration can be lowered to 0.5 per cent without any pronounced effect on the color formation.

Concentration of hydrochloric acid. A decrease in the concentration of hydrochloric acid causes a decrease in color formation. When the reagent is composed of 70 per cent thymol solution and 30 per cent concentrated hydrochloric acid no color is formed.

Stability of the color. If the test tubes after heating are kept at room temperature a small increase in color is observed.

In an ice water bath, however, the color is stable for at least 30 minutes after the initial 20 minutes cooling period.

Table II. *Color equivalents of different classes of sugars.*

Sugar	Amount μg	Optical density	Fructose equivalent μg
Sorbose ...	500	0.395	439
Inulin	100	0.095	111
Sucrose	500	0.230	270
Xylose	5000	0.008	less than 10
Ribose	5000	0.005	—»—
Glucose ...	5000	0.005	—»—
Galactose...	5000	0.004	—»—
Mannose ..	5000	0.005	—»—

Table IV. *Stability of the color.*

	Optical density Room temp. +16°C	Ice water bath ±0°C
15 min.	0.080	0.075
30 »	0.090	0.075
60 »	0.085	0.080
90 »	0.090	0.085
2 hours	0.092	0.085
3 »	0.095	0.090
4 »	0.100	0.095
24 »	0.175	—

Blank values. When read against alcohol the blanks give practically no light absorption at 505 mμ.

DETERMINATION OF FRUCTOSE IN URINE

Attempts to determine fructose in urine directly with the thymol reagent were unsuccesful chiefly because of the presence in urine of coloring matter which gave rise to interfering colors. The removal of the coloring matter from urine without any loss of fructose is most easily accomplished by treatment of the urine with charcoal in acetic acid as described by Roe (1934).

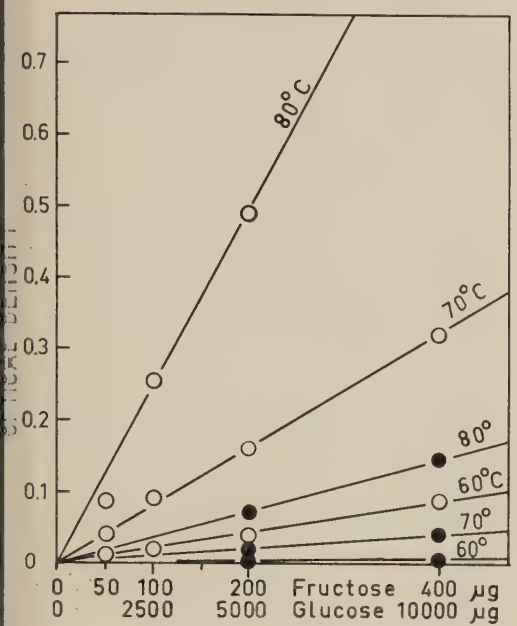

Fig. 3. The effect of temperature on the color formation in fructose and glucose solutions.
-o-o-o-o- fructose solutions.
-●-●-●- glucose solutions.

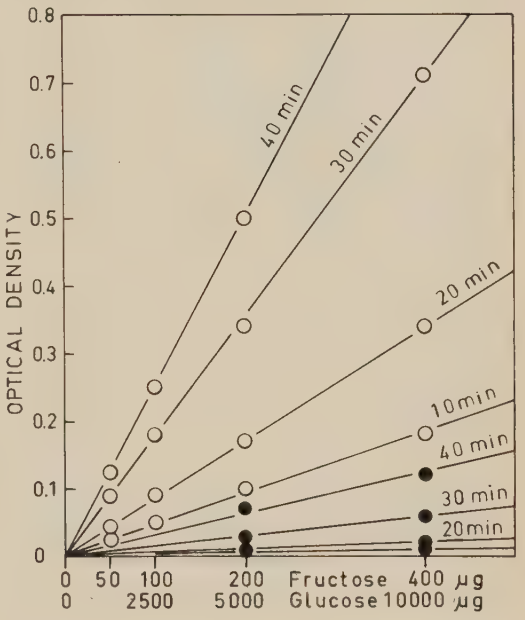

Fig. 4. The effect of heating time on the color formation in fructose and glucose solutions.
-o-o-o-o- fructose solutions.
-●-●-●- glucose solutions.

Another complication in the determination of fructose in urine is the necessity of having the sample dissolved in alcohol. Thus the dilution of the urine, at first with acetic acid and then with alcohol, causes the lower limit of measurements of the fructose concentration in the urine to be 0.5 mg per ml.

METHOD.

Reagents

1. The reagents for the fructose determination are the same as those described in section 1.
2. Charcoal. The charcoal is prepared mainly according to Roe (1934). 100 g charcoal (Hopkins & Williams, London) is suspended in approximately 1 litre of 10 per cent acetic acid. After being stirred for five minutes the charcoal is filtered and washed with about 2 litres of water. Reactivation is accomplished by heating the charcoal to redness for 15 minutes.
3. 2.5 per cent acetic acid.

Procedure

5 ml of urine containing between 0.5 and 8 mg of fructose per ml is mixed with approximately 0.5 g of charcoal. The mixture is shaken and left for 14 minutes. 5 ml of 2.5 per cent acetic acid is then added. After 5 minutes the mixture is filtered. 1 ml of the water-clear filtrate is taken and diluted to 10 ml with 9 per cent ethanol. The fructose concentration is then determined according to the procedure described in section 1.

RESULTS AND DISCUSSION

Recovery of fructose from urine. Different amounts of fructose were added to urine and the fructose concentration determined.

Table V. *Recovery of added fructose from urine containing different amounts of glucose (added). Mean values from triple determinations.*

Added fructose in per cent	Added glucose in per cent	Recovered fructose in per cent	Recovery in per cent
0.10	0.50	0.10	100
0.10	1.00	0.10	100
0.20	1.00	0.20	100
0.20	2.00	0.20	100
0.20	4.00	0.21	105
0.20	5.00	0.21	105

Table VI. *Recovery of added fructose from blood. Mean values from triple determinations.*

Added fructose in mg per cent	Recovered fructose in mg per cent	Recovery in per cent
50	52	104
100	106	106
200	200	100
300	306	102

Since urine from normal non-fasting individuals usually contains small amounts of fructose urine from fasting individuals or urine treated with yeast for 60 minutes at 37° C was used for these recovery experiments. The recovery was 100 per cent.

The determination of fructose in urine containing different amounts of glucose is shown in Table V.

Since the method was primarily developed for the determination of fructose in urine from diabetics, the possible interference of the ketone bodies in such urine was also investigated. No interference from ketone bodies was noted.

It appears from these experiments that it is possible to determine fructose (ketoses) specifically even if the glucose concentration is as high as 50 mg per ml and in the presence of large amounts of ketone bodies. It appears to be possible to increase the sensitivity of the method by means of an independent assay for glucose to permit calculation of the approximate amount of false response in the fructose determination.

DETERMINATION OF FRUCTOSE IN BLOOD

The determination of fructose in blood with the thymol reagent requires the removal of the proteins. Of the various protein precipitants, *e. g.* alcohol, heat coagulation, tungstic acid and trichloracetic acid, the last mentioned appeared to be the best. Since further dilution of the sample with alcohol is necessary, the lower limit of the method for the determination of fructose in blood is at a concentration of about 0.5 mg per ml.

METHOD

Reagents

1. The reagents for the fructose determination are the same as described in section 1.
2. 50 per cent trichloracetic acid.

Procedure

3 ml of blood, taken by vein puncture and 0.75 ml of 50 per cent trichloracetic acid are mixed in a test tube with the aid of a small glass rod.

After 10 minutes the mixture is filtered.

1 ml of the water-clear filtrate is diluted to 10 ml with 96 per cent alcohol. From the solution 1 ml is taken and the fructose determined as described in section 1.

RESULTS

The recovery of different amounts of fructose from blood is seen in Table VI.

SUMMARY

A colorimetric method for the determination of fructose and other ketoses or ketose containing oligo- and polysaccharides with thymol-hydrochloric acid reagent is described. In the range 25—400 μg per ml, fructose can be determined specificially even in the presence of up to 5 mg per ml of glucose and other aldosugars. The method has also been adapted to the determination of fructose in urine and blood.

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CORRECTIONS

P. 224, column 1, line 12 from above, read:
.... development of color with glucose heating was ....
P. 224, column 2, line 22 from above and p. 225, line 2 in text of fig. 2, read:
.... 86 per cent alcohol ....
P. 227, column 2, line 11 from below, read:
.... left for 15 minutes ....
P. 227, column 2, line 7 from below, read:
.... 96 per cent ethanol ....

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THE PRESENCE OF AMINO ACIDS IN URINARY CALCULI

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(Received for publication May 14, 1956)

By F. HEIJKENSKJÖLD and H. MÖLLERBERG

Amino acids in the urine were demonstrated as long ago as 1860 by Frerichs who found crystals of tyrosine and leucine in cases of liver necrosis. It is now a well-known fact that amino acids are normally excreted in measurable amounts in the urine. There is, however, a considerable qualitative and quantitative variation in the amino acid patterns in normal as well as in pathological conditions (Dent, 1946; Stein, 1953).

Certain disturbances of the protein metabolism of the organism may *e. g.* lead to a highly increased excretion of cystine (Dent, Heathcote & Joron, 1954) which favors the formation of cystine calculi in the urinary tract. In other pathological conditions calculi of a different composition are formed — *e. g.* uric acid, calcium oxalate, calcium phosphate, triple phosphate. Whether these urinary calculi contain amino acids has — as far as we know — not yet been investigated. This paper is a preliminary report on the presence of amino acids in urinary calculi of different origin.

MATERIAL AND METHODS

The material comprised thirteen urinary calculi. The qualitative chemical analyses and the amino acid determinations were made by different investigators. The calculi were carefully rinsed in saline to remove contaminations (blood clots *etc.*). The size, color and other physical properties were noted. The calculi were then dried and thoroughly ground into a powder in an agate mortar. The powder was divided into two parts, one for the micro-chemical analyses and the other for the amino acid determinations.

The *micro-chemical analysis* was carried out according to a method suggested by G. Hammarsten (1940).

The *qualitative chromatographic amino acid determination* was made as follows:

About 100 mg of each pulverized calculus were transferred to 100 ml flasks. 1 ml distilled water was added to each flask in order to get the powder thoroughly wetted. The flasks were then filled with acid acetone (1 per cent HCl), stoppered and shaken and left overnight at room temperature. The mixture was then filtered and evaporated *in vacuo* to a small volume at 70°—80° C. When the acetone had been removed, the temperature was lowered to about 40° C. The evaporation was continued to dryness. The residue, containing among other things acetone-soluble amino acids, was dissolved in 0.1—0.5 ml distilled water and applied to chromatograms.

One-dimensional descending chromatography was made, using the butanol phase of a mixture of butanol, acetic acid and distilled water (v/v/v 4:1:5).

The chromatograms were run on Munktell OE paper at room temperature for fifteen hours. After drying at 25° C, the papers were dipped into a 0.2 per cent acetone solution of ninhydrin. After renewed drying, the chromatograms were left at room temperature for 48 hours. The color reaction was then fully developed. The color spots were

ked by treatment with 1 per cent cupric nitrate 95 per cent ethanol which gave distinct d spots on a white background after drying.

The different amino acids were satisfactorily parated. Thus the distance between the cystine d leucine spots was about 30 cm. A test solution th known amino acid composition was always n together with the unknown samples for identi- cation. By this method it was possible to separate e amino acids with the exception of the following irs: lysine and histidine — alanine and valine methionine and tryptophan — leucine and iso- ucine which run at the same velocity in couples. ne spots corresponding to these amino acids were sumed to contain both amino acids.

To make sure that no peptides had been extracted the acid acetone, part of the residue remaining ter the acetone procedure was hydrolysed with N HCl at 100° C for 10 hours, evaporated to yness *in vacuo,* dissolved in water and chromato- aphed. The hydrolysed samples gave the same ots as the non-hydrolysed ones. It was thus con- lered probable that no mentionable amounts of ptides had been extracted from the powdered lculi.

RESULTS

The results of the analyses are shown in e table. Calcium, phosphate, magnesium, mmonium and oxalate ions were demon- rated in the most common type of calculus. arbonate was detected in one single cal- ulus.

Amino acids were found in all analysed lculi but the one consisting of uric acid. he cystine calculus in the material con- ined no demonstrable amounts of inorganic ubstances. It was, however, possible to dentify lysine and histidine together with ystine.

Hydrolysis of the cystine calculus yielded n chromatography six additional spots as mpared with the unhydrolysed sample. ome of the spots were weakly colored treaks on the chromatograms which are often seen on analysis of impure substances. Analyses of pure cystine gave the same result after hydrolysis. Whether this result is due to contamination with oxidizing substances is not yet clear. Van Halteren (1951) and Matsuo (1953) have shown that oxidation of cystine and methionine results in reaction products difficult to identify. Paper electrophoresis (veronal buffer at pH 8.6, ionic strength 0.1 μ) of the cystine calculus dissolved in ammonia gave only two spots comparable with those obtained before hydrolysis. From these experiments it might be assumed that the cystine calculus contained only cystine, lysine and histidine, in spite of the appearance of the different spots on chromatography after hydrolysis.

DISCUSSION

The demonstration of amino acids in urinary calculi raises the question whether there is any correlation between the mineral constituents and the amino acid composition of the calculi.

The table indicates that the most common inorganic constituent in calculi of ordinary type was calcium. Magnesium and ammonium were less frequent. The most common anions were phosphate and oxalate. It is of interest to note that the calculi numbers 8 and 10, consisting of pure calcium oxalate, contained lysine and histidine which were not demonstrable in the other common calculi. Cystine, arginine, glycine, threonine, alanine, valine, leucine and isoleucine were the most frequently occurring amino acids in the calculi which verifies the observations on the presence of free amino acids in normal urine made by Dent (1946) and Stein (1953). A plausible explanation of the pre-

Table. *Composition of urinary calculi.*

				Qualitative chemical analyses								Amino acids									
Calculus No.	Type	Age	Sex	Calcium	Magnesium	Ammonium	Phosphate	Oxalate	Carbonate	Uric acid	Cystine	Cystine	Lysine + Histidine	Arginine	Serine	Glycine	Threonine	Alanine + Valine	Tyrosine	Methionine + Tryptophan	Leucines
1	Bladder calculus	65	♂	+				+				+		+		+	+	+			+
2	Renal calculus	47	♀	+			+	+				+		+		+	+	+	+		+
3	Prostatic calculus.....	67	♂	+	+		+					+		+		+	+	+			+
4	Renal calculus	49	♀	+	+	+	+	+				+		+		+	+	+			+
5	Bladder calculus	27	♂	+	+	+	+		+			+		+		+	+	+			
6	»	52	♂	+	+		+					+		+		+	+	+			+
7	Renal calculus	47	♂	+	+	+	+					+		+		+	+	+			+
8	»	44	♀	+				+				+	+	+		+	+	+			
9	»	26	♂	+	+	+	+	+				+		+		+	+	+			+
10	»	59	♂	+				+				+	+	+	+	+	+	+	+	+	+
11	»	1	♂							+											
12	»	15	♀	+	+		+					+				+	+	+			+
13	»	42	♀								+	+	+								

ence of amino acids in the calculi seems to e that the amino acids have been absorbed nto the growing calculi and thus have successively been imbedded.

The question as to whether the amino acid ontent of the urine is a pathogenetic factor n the formation of the inorganic calculi is till unsettled. Conditions with generally ncreased excretion of amino acids in the rine, such as starvation, fever and stress, ave not shown any increased tendency to formation of common urinary calculi. Dent, Heathcote & Joron (1954) have shown, however, that the formation of cystine calculi is due to a greatly increased excretion of cystine, ornithine, lysine and arginine in the urine. This observation is compatible with the presence of lysine and histidine together with cystine in the cystine calculus number 13 in our material. Amino acid determinations on the urines from the patients operated on for the calculi were not made.

SUMMARY

Paper chromatography for amino acid determination and qualitative inorganic chemical analysis have been used on thirteen urinary calculi. About ten amino acids in different combinations have been demonstrated. The correlation between amino acids and inorganic constituents is briefly discussed.

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Scandinav. J. Clin. & Lab. Investigation 234—236, 8, 1956.

ON THE IODIDE-OXIDISING SUBSTANCE IN URINE

II. CHEMICAL STUDIES

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(Received for publication May 23, 1956)

This work was prompted by a report by Fishberg (1948), who found an iodide-oxidising substance in the urine of patients suffering from enterogenous cyanosis or from rheumatic fever. She identified it as the benzoquinoneacetic acid. We investigated about 2300 samples and found the iodide-oxidation fairly common (2.2 per cent), especially in the diabetic urines (41.4 per cent). Sometimes this substance could be formed afterwards in the urine. An account of these studies is published separately (Hallman & Kulonen, 1954). This report deals with chemical experiments which showed that this substance is usually not benzoquinoneacetic acid. The possibility of nitrites or peroxides was therefore investigated.

EXPERIMENTAL

For the *determination* we used the procedure of Valeur as suggested by Fishberg. To 10 ml urine were added 5 ml of 10 per cent (by volume) sulphuric acid and 5 ml of 10 per cent potassium iodide. A drop of starch solution was added as indicator. The color doet not develop instantly and as a rule the titration was performed after 10 minutes, using .01 N sodium thiosulphate.

At first we attempted to follow the procedure of Fishberg for the *isolation*. Urine was acidified with hydrochloric acid to pH 3.5 and shaken in a separatory funnel with an equal volume of peroxide-free ether. The oxidising agent could not be extracted from neutral or alkalinized urine. The oxidising capacity was exhausted from the urine by repeated extraction. The ether extract was dried with exsiccated sodium sulphate and evaporated in a stream of nitrogen. The yellow residue was soluble in chloroform and crystallised by evaporation, but slowly lost the oxidising ability. (From a normal urine only some pigment and amorphous material was obtained.) The melting point of the residue was 103—106° C. The solubility in chloroform is not in agreement with the description of the benzoquinoneacetic acid by Mörner (1912). The residue gave no distinct color with benzidine acetate, *p*-phenylenediamine, sulphuric acid, sodium hydroxide or ferric chloride. A reduction with stannous chloride was unsuccessfully attempted to demonstrate the formation of homogentisic acid or other phenols with ferric chloride. Liebermann-Burchardt's and Salkowski's reactions were negative.

The next approach was to use steam distillation. The oxidising agent was volatile but was rapidly destroyed by heating in the acid environment (pH 3). The resulting inactivated sample reduced iodine, as did the distillate of normal urine. The ultraviolet absorption spectra of both distillates were identical.

The oxidising substance was thermostable on the evaporation of the soda-alkaline solution. If a neutral or ammonical solution was evaporated the oxidising capacity was lost.

Since the destruction in an acid environment is not in agreement with the quinoid concept we tried to obtain information on the presence of *peroxides*. We could not secure any positive proof. The active

reparations did not oxidise benzidine acetate in he presence of hemin. The occurrence of hydrogen eroxide was tested using chromic acid, which was ot oxidised by the active preparations. These eactions do not necessarily exclude the peroxide oncept, since only relatively labile peroxides libe- ate hydrogen peroxide.

The next consideration was for the products of *utooxidation* of unsaturated compounds, which ould yield carbonyl derivatives in degradation. ishberg presents the precipitate formation with 4-dinitrophenylhydrazine as evidence for quinoid ructure. However, the primary hydrazines are sually oxidised by quinones. Carbonyl compounds e also normally present in the steam distillates the urine.

To elucidate whether any carbonyl compounds ere formed when the oxidative capacity was de- royed by heating (for two minutes in .1 N hydro- loric acid), their presence was tested by addition fuchsin-sulphurous acid, which formed stronger olor in the inactivated sample. To obtain more curate information, one-third by volume of cold, esh, saturated solution of 2,4-dinitrophenylhydra- ne in 2 *N* hydrochloric acid was added. As com- ared with the control sample, which was not eated, the color deepened and a precipitate formed. he hydrazones were extracted with ether, which turn was extracted with 5 per cent sodium car- onate solution to separate the acid and neutral ydrazines. Both fractions were darker in the activated sample. A more detailed analysis was ade with paper chromatography (Kulonen, Car- n & Ruokolainen, 1952). It was found that a w slightly acid carbonyl compound (which was ot phenylpyruvic acid had been formed. The spot as soluble in sodium hydroxide but not in hydro- loric acid, thus excluding dinitroaniline. The ydroxyphenylpyruvic acid was not available as ference but it should give a color with ferric loride. Two carbonyl compounds would actually e expected from a degradation of an auto-oxidised nsaturated fatty acid. No neutral carbonyl com- ound was observed in chromatography (Kulonen, 955).

The possibility that this carbonyl compound was rmed as an artefact by the oxidising principle as tested by boiling normal urine acidified to pH 1, with hydrogen peroxide (1 per cent). No corresponding increase was observed in the 2,4-dimitrophenylhydrazones.

Only two groups or organic compounds seem to have an oxidising capacity strong enough to liberate iodine from iodides, namely quinones and peroxides. These experiments do not supply positive evidence for either group. Therefore the presence of *nitrites* (suggested by prof. P. E. Simola) was investigated further. Nitrites liberate iodine in very small concentrations, are labile and ether-soluble in an acid environment, and are known to occur in the urine, at least if infected. The presence of nitrites was tested using the diazotization of sulphanilic acid and coupling with α-naphthylamine (Feigl, 1947). Oxidising urine samples, normal urine, normal urine with addition of nitrite (1—3 per cent), nitrite solution and ether extracts from these were tested. A qualitative correlation with the iodide-oxidising power was found but it was not consistent.

The presence of nitrites was investigated further by paper chromatography (Lederer, 1953). The first attempts were made with butanol/pyridine/1.5 *N* ammonia (2:1:2) as solvent, but the pyridine consumed iodine. Better results were obtained with butanol, saturated with 1.5 *N* ammonia. The paper was allowed to dry and was sprayed with a solution containing potassium iodide, sulphuric acid (5 per cent of each) and starch. The blue spots appeared instantly but after some hours the background darkened also. The iodide-oxidising agent was used as an ether extract but a drop of *N* sodium hydroxide had to be evaporated beforehand on the intended location of the spot to "fix" the ether extract on the paper. The chromatograms were interpreted to show that the oxidation in some cases is due to nitrites, but the facts do not warrant the conclusion that it is the sole factor.

The possibility of formation of this oxidative agent *in vitro* caused us much concern. Fifteen negative samples of diabetic urines were incubated for 4 hours at 37° C, but no oxidising activity appeared. In the second series three positive samples were divided into three parts each, one was kept at room temperature, the second incubated under a layer of liquid paraffin and the third was treated with a stream of nitrogen. No essential

change appeared in two hours. One series of fifteen negative diabetic urine samples was titrated back with iodine but the consumption remained unchanged for four hours at 37° C. However, after 18 hours' incubation at 37° C three strongly positive samples appeared in a series of 33 samples. Many positive cases were recorded immediately after voiding to prove the formation also *in vivo*.

COMMENTS

In spite of differences in the results we do not doubt the accuracy of Fishberg's analyses, which were confirmed by the preparation of a homogentisic acid derivative. We only wish to point out that other possibilities for the iodide-oxidising capacity of urine also exist.

In the old literature the occurrence of hydrogen peroxide in fresh urine has been cited (Schönbein, 1864), but the observation has not been followed since. There are also some studies on the metabolism of fatty acid peroxides which are deposited in the adipose tissue but the oxidising capacity disappears rapidly.

It is generally known that urine infected with *B. coli-* or *proteus*-type of microorganism may contain nitrite, but Varaday & Szanto (1940) claim that nitrite is excreted by the kidneys in some grave diseases, mostly in malignant tumors. Their samples were obtained by catheterisation. In the diabetic cases, presented in the first part of this report, there was no clinical evidence of urinary infection. There was no correlation with the sugar content of the urine but there did exist some inverse correlation with the presence of acetone bodies. These facts do not support the idea that the nitrite was formed *in vitro*.

The formation of the above-mentioned carbonyl compound might be accounted for by oxidation *in vitro*. Also the crystallised fatty material obtained by ether extraction may be regarded as a "carrier" for the oxidative agent.

SUMMARY

The iodide-oxidising capacity of urine was studied chemically and experiments on the enriched preparation and its properties are presented. Benzoquinoneacetic acid, peroxides and nitrites were considered. At least nitrite was present.

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THE USE OF BUFFERS IN BLOOD COAGULATION STUDIES

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The requirements to be fullfilled for buffers suited for coagulation studies are the following: a) maximal buffering ability between pH 7 and 8, b) absence of calcium binding properties, c) absence of accelerating or inhibitory effects on the clotting mechanism. Due to these requirements only a few buffers are commonly used. In this laboratory we also maintain that the ionic strength should be adjusted to that of physiological saline (Owren, 1947).

Astrup (1944) was the first to point out that it is necessary to take the ionic strength into consideration in coagulation studies because of its pronounced influence on the velocity of enzyme reactions. This is particularly important in clotting studies where the velocity of clotting most often forms the basis for quantitative assay of various factors. As a consequence of this Owren (1947) adjusted the ionic strength of veronal buffer to that of 0.9 per cent sodium chloride, regarding this as a prerequisite for reproducible results.

To get reliable results with hypoionic buffers implies that the volume of buffer must always be in the same ratio to the total mixture volume, which is difficult to achieve. Use of buffers with different ionic strength also makes it difficult to compare results from one laboratory to another.

In spite of these authors' experiments both imidazole (Kirby & Neuberger, 1938) and veronal buffer (Michaelis, 1932) are still most commonly used in their original form, *i. e.* with low ionic strength. For example, most workers using imidazole buffer refer to the paper of Mertz & Owen (1940). These authors did not maintain constant ionic strength (Astrup, 1944).

Biggs, Eveling & Richards (1955) compared glyoxaline (imidazole), methylglyoxaline, veronal, and collidine buffers in the antihemophilic globulin assay based on the thromboplastin generation test. They found that veronal and collidine buffers were inhibitory. However, they did not state the ionic strength of their buffers.

A further abuse of buffers is that they are expected to correct the pH of reagents with widely differing pH values. In biological fluids buffers should not be used to correct pH, but merely to maintain it at a given level. This is particularly true for reagents having a marked buffer capacity of their own, such as plasma and serum.

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In this investigation we have compared the effect of imidazole, veronal, and tris-(hydroxymethyl)aminomethane buffers in different clotting systems, taking the ionic strength into consideration. Their buffer capacity is also evaluated in a practical test.

MATERIALS AND METHODS

Citrated plasma was prepared from blood drawn in 1/10 volume of sodium citrate dihydrate 3.13 per cent.

Substrate plasma for thrombin estimation was prepared from oxalated plasma (one part 2.5 per cent potassium oxalate monohydrate to nine parts of blood) by barium sulphate (Baker) adsorption (100 mg/ml plasma) and dialysing against 0.9 per cent saline for 24 hours.

Dowex plasma was prepared from blood passed once through a 5 cm column of Dowex — 50 ion exchange resin, 20—50 mesh. (Resin rejuvenated to sodium cycle by 10 per cent NaOH followed by 18 hours rinsing with continually dripping 0.9 per cent NaCl)

Thrombin solution was made by dissolving bovine thrombin (Haemoclaudan-Adrenon, Leo, Copenhagen) in physiological saline of pH 7.35.

Imidazole buffer. A stock solution was prepared by adding 14.8 ml 0.1 N HCl to 25 ml 0.2 M imidazole and diluting to 100 ml with distilled water. pH: 7.35, $\mu = 0.0148$.

Veronal buffer. The stock solution was made by adding 21.5 ml 0.1 N HCl to 25 ml 0.114 sodium diethylbarbiturate and diluting to 100 ml with distilled water. (Same molarity as in Owren's modification of Michaelis' buffer, but without sodium chloride added.) pH: 7.35, $\mu = 0.0285$.

Tris(hydroxymethyl)aminomethane buffer (Sigma 7—9 buffer, Sigma Chemical Company, St. Louis, Mo., U.S.A.). The stock solution was prepared by adding 42.5 ml 0.1 N HCl to 25 0.2 M Sigma 7—9 buffer and diluting to 100 ml with distilled water. pH 7.35, $\mu = 0.0425$.

Cephalin was prepared by a modification of Milstones technique (Rapaport, 1954).

Thromboplastin was made according to Owren (1947).

pH was measured electrometrically with a Radiometer pH-meter, Copenhagen, accuracy ± 0.05 pH unit.

Control of ionic strength. The formula $\mu = \frac{1}{2} \Sigma mz^2$, where m is the molar concentration of each single ion and z the valence, has been used for calculating ionic strength (μ). To obtain correct values with this equation, the dissociation must be complete. This condition is not fullfilled here, because of the relatively high electrolyte concentrations. The figures, therefore, are approximate, but since only the relative figures are of interest in this study, we have assumed complete dissociation.

The plain buffers as described above are hypoionic with respect to 0.9 per cent sodium chloride. To show the influence of varying ionic strength in the clotting systems and at the same time compare the effect of the buffers at equal ionic strength, known quantities of sodium chloride were added to the buffers up to $\mu = 0.154$ and the mixtures tested after each addition. From the formula it can be calculated that isoionicity with 0.9 per cent saline requires the addition of 0.81, 0.74 and 0.66 per cent sodium chloride to imidazole, veronal and Sigma 7—9 respectively.

The following clotting systems were used:

Thrombin time.

0.2 ml substrate plasma.
0.2 » saline or buffer, varying ionic strength.
0.2 » thrombin solution.

Quick thromboplastin time.

0.2 ml saline brain thromboplastin.
0.2 » buffer or saline, varying ionic strength.
0.2 » fresh, normal, citrated human plasma pH 7.35.
0.2 » $CaCl_2$, 0.03 M.

Cephalin time.

0.2 ml cephalin, optimal concentration.
0.2 » buffer or saline, varying ionic strength.
0.2 » citrated plasma as above.
0.2 » $CaCl_2$, 0.03 M.

Prothrombinase generation was examined in a system patterned after the thromboplastin generation test (Biggs & Douglas, 1953) as follows:

Incubation mixture: Equal parts of:

a) Normal, oxalated, barium sulphate adsorbed (100 mg/ml) human plasma, pH 7.35 (source of hemophilia A factor and proaccelerin). Dilution 1:10 in 0.9 per cent saline, pH 7.35 (Fig. 2, 3) or 1:5 (Fig. 4).

b) Hemophilia B factor. (Purified by a modification of the method of White, Aggeler, & Glendening, 1952). Dilution 1:5 in saline, pH 7.35.

c) Cephalin. Optimal dilution in saline, pH 7.35 (1:200).

d) Calcium chloride, 0.03 M.

e) Two parts of saline or buffer of varying ionic strength.

Final buffer concentration in mixture 1/3 [1].

Substrate: Normal, decalcified (Dowex-50 ion exchange resin), human plasma, pH 7.35.

Technique: The modification of Duckert *et al.* (1954) was followed.

Reporting of results: One series of curves (imidazole) are presented as an example in Figure 2. In an effort to compare similar series for other buffers, two aspects of each curve were plotted. *The minimal clotting time* is the average of the three shortest clotting times for each curve. *The incubation time* was arbitrarily defined as the duration of incubation (after the addition of calcium) necessary to produce a prothrombinase capable of clotting the substrate plasma in 25 seconds (Figure 3).

Throughout the experiments pH was rigidly controlled, so that the only variable in each experiment should be the ionic strength. Saline was used as a standard of reference in comparing the buffers.

[1] In practice one usually dilutes all reagents in buffer, but this ratio was used to demonstrate that even with the minimum buffer amounts above one must control ionic strength.

RESULTS

Effect on different clotting systems.

The thrombin-fibrinogen reaction. From Fig. 1 it is clear that this reaction is profoundly influenced by variations in ionic strength. Thus, when the ionic strength increases towards that of physiological saline in the saline or buffer included in the system, a marked inhibition of the reaction is found. (26 to 51 secs., which corresponds to about 100 per cent difference in thrombin units). It seems more logical, however, to take the opposite point of view, namely that the very low ionic strength of the plain buffers is not physiologic and, therefore, unphysiologically *accelerates* the reaction.

The Quick thromboplastin time. A similar trend is noted here. However, the differences in clotting times are very slight.

The cephalin (partial thromboplastin) time. This system reflects in a similar fashion the influence of ions on clotting. Here the clotting time is accelerated from 65 seconds to 54 seconds by the actual decrease of the ion concentration.

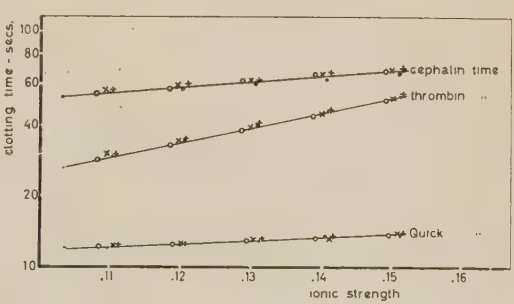

Fig. 1. Comparison of buffers at controlled ionic strengths in three different clotting systems.
● — saline.
o — imidazole buffer.
× — veronal buffer.
+ — Sigma 7—9 buffer.

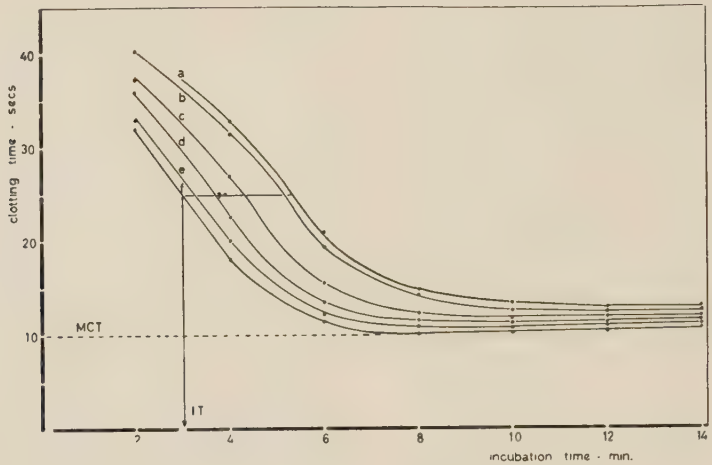

Fig. 2. Acceleration of prothrombinase generation (thromboplastin generation test) by use of hypoionic buffer solutions (imidazole).
MCT minimal clotting time: curve f.
IT incubation time: curve f (see text).
Incubation mixture constant except for NaCl concentration of buffer.

a — 0.81 per cent NaCl added
b — 0.72 » »
c — 0.54 » »
d — 0.36 » »
e — 0.18 » »
f — 0.00 » »

Prothrombinase generation. Decrease of ionic strength below that of 0.9 per cent saline clearly has an accelerating effect on the rate of prothrombinase formation. This is reflected to a lesser degree by the maximum level of activity reached where a slight difference comparable to the Quick time is seen. However, if clotting time is converted to per cent prothrombinase activity (from a dilution curve at maximal activity) this three second difference represents 30 per cent or more change in prothrombinase activity.

Effect of buffers used on clotting activity.

As is evident from the figures, no difference between veronal, imidazole, and Sigma 7—9 buffer systems was noted at comparable ionic strength. This is apparent in the two tests most sensitive to variations in ionic strength, the thrombin system (Fig. 1) and the prothrombinase generation system (Fig. 4).

The buffer capacity.

To illustrate in a practical manner the buffer capacity, increasing amounts of each

Table I. *Comparison of buffer capacity of imidazole, veronal, and Sigma 7—9 buffer.*

ml buffer added to 4 ml plasma	pH		
	Imidazole (7.35)	Veronal (7.35)	Sigma (7.35)
0	8.01	8.00	8.00
1	7.72	7.76	7.75
2	7.62	7.68	7.64
3	7.60	7.61	7.60
4	7.56	7.58	7.57
5	7.53	7.55	7.54
6	7.50	7.52	7.52

buffer were added to 4 ml of plasma pH 8 and the pH of the mixture measured.

It is apparent from Table II that the difference in buffer capacity between the three buffers is only slight within the range tested. Although imidazole is somewhat superior to the other two in this respect, the difference is so negligible that it offers no basis for chosing between them.

In another experiment it was shown that plasma of pH 8 needed at least 5 times its volume of buffer to obtain the pH of the buffer.

DISCUSSION

From these studies we conclude that either imidazole, veronal or Sigma 7—9 are good buffers for use in clotting studies provided sodium chloride is added.

With the use of these buffers in their original form, both prothrombinase formation and the thrombin-fibrinogen reaction are accelerated due to the low ionic strength of the buffers. To meet the demand for buffers which are iso-ionic with all plasmas is impossible since the ionic strength of a given plasma will vary with the anticoagulant used, the hematocrit, and with factors in the plasma itself. It seems most reasonable to use buffers with the same ionic strength as 0.9 per cent NaCl since this closely appro-

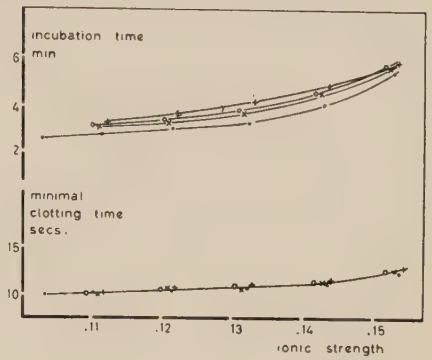

Fig. 3. Prothrombinase generation. Influence of buffers at controlled ionic strengths on minimal clotting time and incubation time (see text and Fig. 2). Markings as in Fig. 1.

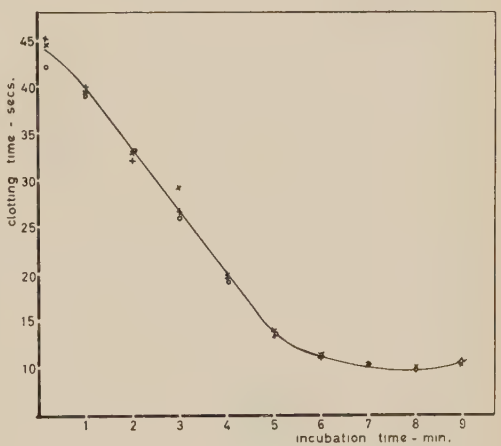

Fig. 4. Comparison of buffers in prothrombinase generation at controlled ionic strengths. Each point represents a complete curve (see text). Markings as in Fig. 1.

aches the ionic strength of plasma and has the advantage of common usage. This allows the mixing of buffer and plasma in varying proportions with minimal variability in the ionic strength of the mixture.

Two points must be emphasized. First, it is not valid to compare buffer systems with different ionic strengths. Second, it is essential to adjust all reagents to the pH desired in the system. The addition of buffer may then be expected to maintain this pH constant. If this is not done, the pH will be somewhere between that of the buffer and that of the reagent.

SUMMARY

1. The effect of imidazole, veronal, and tris(hydroxymethyl)aminomethane (Sigma 7—9) buffers were compared in several coagulation systems.
2. At comparable ionic strengths the three buffers gave similar results.
3. Buffers used in coagulation work should be adjusted to the same ionic strength as physiological saline.
4. Buffer capacity varied slightly within the actual range (pH 7—8), but not sufficiently to be of practical importance.
5. From the standpoint of all round utility (solubility, cost *etc.*) the tris(hydroxymethyl) aminomethane buffer is preferable.

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Technique and Practical Problems

Scandinav. J. Clin. & Lab. Investigation 243—249, 8, 1956.

THE EFFICIENCY OF BUTANOL EXTRACTIONS OF SERUM ORGANIC IODINE COMPOUNDS

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(Received for publication December 20, 1955)

Since Leland & Foster in 1932 described the use of n-butanol as a solvent for the extraction of thyroxine from hydrolyzed thyroid tissue, it has been widely employed for investigating the iodized amino-acids in the thyroid gland and has proved to be very helpful.

Several investigators have used n-butyl alcohol for the fractionation of the iodized compounds in blood (Trevorrow, 1939; Elmer, Luczynski & Scheps, 1934; Basset, Coons & Salter, 1941; Wilmanns, 1943). Since then it has been a most valuable tool for the study of the nature of the circulating thyroid hormone (Taurog & Chaikoff, 1948; Gross & Pitt-Rivers, 1952).

The extraction of plasma or serum with n-butyl alcohol is usually carried out for two main purposes:

1) The chemical determination of hormonal iodine.

2) The identification of the chemical nature of the plasma organic iodine compounds, after labeling with I^{131}.

In the first case, the serum or plasma is extracted several times with n-butyl alcohol (Taurog & Chaikoff, 1948). The extract thus obtained is freed from iodide by washing with Blau's (1933) alkaline solution. In the washed butanol extract the iodine content is determined micro-chemically.

In the second case, the serum or plasma of animals which have previously received I^{131} in tracer or therapeutic doses is extracted and the radioactive compounds can be identified.

The procedure used for the extraction by different authors differs slightly: in the amounts of sample and n-butyl alcohol used, the pH *etc.*

Since it is of the utmost importance that the iodized organic compounds are quantitatively extracted, we decided to investigate the optimal conditions for the extraction procedure. The results of this study will be reported here.

METHOD

Plasma of rats which had received tracer doses of carrier free I^{131} were extracted several times with n-butyl alcohol. The nature of the radioactive compounds was identified by paper-chromatography in each one of the successive extracts of the same plasma. The procedure was repeated with a human serum to which I^{131} as thyroxine, triiodothyronine and iodide was added.

In this way it was possible to determine the number of extractions necessary to extract the plasma organic iodized compounds completely.

Procedure:

2.0 ml serum or plasma were transferred to a glasstoppered cntrifuge tube. 1 N H_2SO_4 was added dropwise until the pH was 2—3 (Man, Kydd & Peters, 1951).

A certain amount of n-butyl alcohol, equilibrated over an aqueous solution of sodium thiosulfate, was

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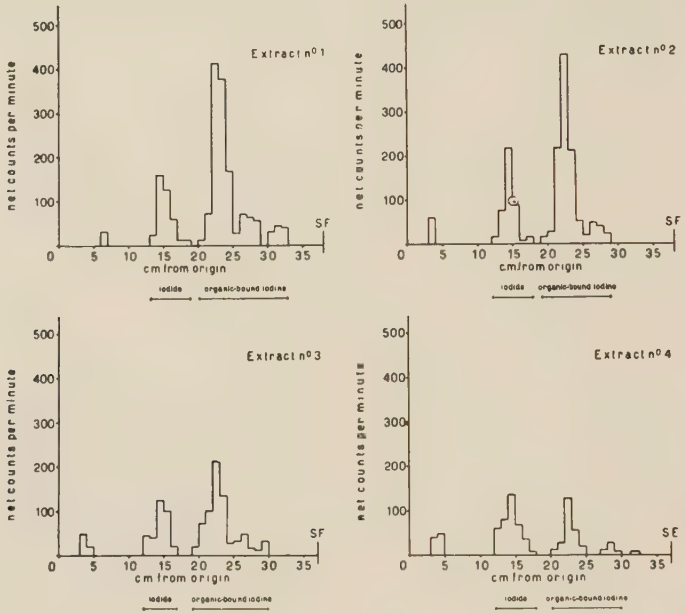

Fig. 1. I^{131} distribution on paper chromatograms of the first four n-butanol extracts obtained using a 2 : 1 relation between solvent and plasma volumes; pH 2—3.
The iodized compounds were separated by descending procedure with n-butanol-dioxane-ammonia as system in the following sequence: di and mono-iodotyrosine, iodide, thyroxine and tri-iodothyronine. A last unidentified peak was often observed. S. F. = solvent front.

added and the mixture thoroughly shaken and then centrifuged at 2500 r.p.m. for about 10 minutes. The supernatant butanol layer was separated by means of a Pasteur pipette and transferred to a centrifuge tube.

The protein precipitate formed was then stirred with a fine glass rod, n-butyl alcohol was again added, washing the rod carefully; the mixture shaken and centrifuged. The supernatant was separated with the same Pasteur pipette, *but transferred* to a different centrifuge tube.

The extraction procedure was again repeated until up to eight extracts were collected in different tubes.

A few drops of a mixture of methyl alcohol and 25 per cent ammonia (4 : 1) were added to the centrifuge tube containing the first butanol extract. It was then concentrated almost to dryness in a water bath at 70° C.

The residue was quantitatively transferred to Whatmann No. 3 chromatographic paper, always using methyl alcohol-ammonia to wash the centrifuge tube. Iodide, thyroxine, tri-iodothyronine, mono and di-iodotyrosine were added as carriers. The chromatogram was run for about 12—14 hours at 26° C, using a descending procedure with n-butyl alcohol-dioxane-ammonia (5 : 1 : 4) as system. The strip was dried, sprayed with 2.5 per cent Na_2CO_3 and stained for phenol groups with diazosulphanilic acid. The chromatogram was not dyed for iodide, to prevent any possible losses arising from the staining procedure.

The initial serum or plasma sample, the protein residue after each butanol extraction and each extract before and after evaporation were counted in a well-type scintillation counter. The chromatograms were counted with the same scintillation counter, using 1 cm wide strips.

RESULTS

Fig. 1 and Table I present the data on the extraction of 2.0 ml of plasma of a rat, which was injected 48 hours before with 10 μc of carrier-free I^{131}. The procedure described above was followed, using 4.0 ml n-butanol for each of the four extractions.

Most of the radioactivity present in the first three extracts appears as organic iodized compounds. Even if most of the radio-

Table I. *Percentage of plasma I^{131} found in each of four extracts and on the corresponding chromatograms, when the relation between n-butanol and sample volume is 2: 1, and the pH 2—3.*

Per cent of sample I^{131}	Extract No. 1	Extract No. 2	Extract No. 3	Extract No. 4	Total
Extracted	22.1	19.1	11.2	6.4	59.0
Non-extracted					34.5
Present on the corresponding chromatogram as iodide ..	4.4	4.7	4.3	4.4	17.8
Present on the corresponding chromatogram as organic-bound iodine	16.3	13.7	8.3	2.7	41.0
Recovered (found on chromatograms + non-extracted)					93.3
Protein-precipitable (with Somogyi's reagent)					45.0

ctivity on the chromatogram of the fourth xtract is due to iodide, 2.7 per cent of the ctivity of the plasma sample appears in the yroxine spot.

When 4.0 ml of n-butyl alcohol are used or 2.0 ml of sample, the extraction of the rganic iodized *compounds is* not complete fter the procedure has been repeated three mes.

Therefore it was tested whether a better xtraction rate could be obtained by increasing the amount of butanol.

In the next experiment 2.0 ml of plasma f a rat fed a low iodine diet for 12 days, njected 24 hours previously with 7 μc of arrier-free I^{131} were used.

The extraction procedure already described as followed, using this time 5.0 ml n-butyl lcohol for each of four extractions.

The results obtained are given in Fig. 2 nd Table II.

Most of the radioactivity in the form of rganic compounds is extracted the first vo times. In the third chromatogram only bout 4.0 per cent of the total radioactivity f the sample is found in the thyroxine peak. he activity present in the fourth extract is ue only to iodides.

From these results the conclusion seems to be justified that when 5.0 ml n-butyl alcohol are used for 2.0 ml of sample, the iodized organic compounds are extracted completely after three extractions.

In Table III and Fig. 3 another example is presented.

The plasma of a rat fed for 15 days on Remington diet and injected 24 hours previously with 8 μc of carrier free I^{131} was used.

This experiment was carried out to corroborate the results obtained in the previous one, so the same proportion of n-butyl alcohol to plasma was employed. In order to see whether the radioactivity remaining in the protein residue after four extractions was due to iodides only, the procedure was repeated up to eight times. The last five extracts were pooled and run on one chromatogram.

Evidently the results are in agreement with those of the former experiment. The activity due to organic compounds is extracted mainly the first two times. In the third extract only 3.4 per cent of the total activity of the sample appears in the

Table II. *Percentage of plasma I^{131} found in each of four extracts and on the corresponding chromatograms, when the relation between n-butanol and sample volumes is 2.5: 1 and the pH is 2—3.*

Per cent of sample I^{131}	Extract No. 1	Extract No. 2	Extract No. 3	Extract No. 4	Total
Extracted	47.1	17.1	6.7	3.4	74.3
Non-extracted					23.5
Present on the corresponding chromatogram as iodide ..	7.2	4.4	2.4	2.2	16.2
Present on the corresponding chromatogram as organic-bound iodine	38.6	12.8	3.9	—	55.3
Recovered (found on the chromatograms + non-extracted)					95.0

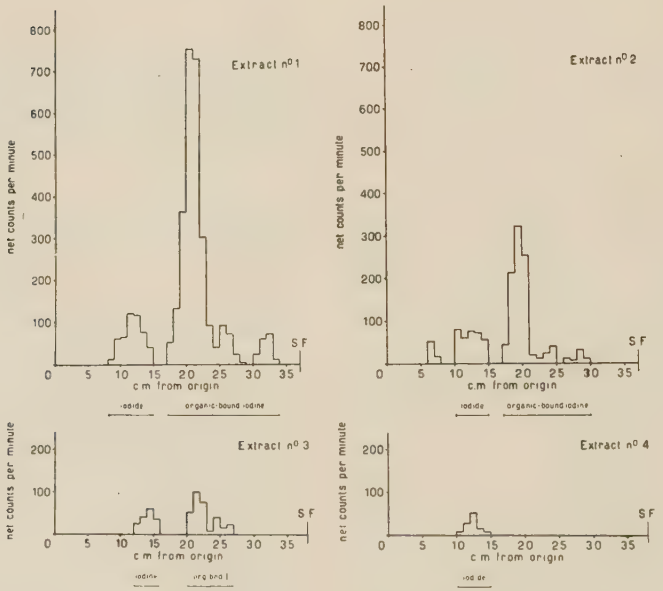

Fig. 2. I^{131} distribution on paper chromatograms of the first four n-butanol extracts obtained using a 2.5 : 1 relation between solvent and plasma volumes; pH 2—3.
The iodized compounds were separated by the same procedure and in the same sequence as in the case of the chromatograms shown in Fig. 1.

Table III. *Percentage of plasma I^{131} found in eight extracts and on the corresponding chromatograms, when the relation between n-butanol and sample volumes is 2.5: 1 and the pH 2—3.*

Per cent of sample I^{131}	Extract No. 1	Extract No. 2	Extract No. 3	Extract No. 4	Total
Extracted	67.0	15.1	5.5	9.0	96.6
Non-extracted					2.8
Present on the corresponding chromatogram as iodide ..	7.7	3.4	2.1	6.2	19.4
Present on the corresponding chromatogram as organic-bound iodine	60.0	11.7	3.6	—	75.3
Recovered (found on chromatograms + non-extracted)					97.5
Protein-precipitable (with Somogyi's reagent)					76.8

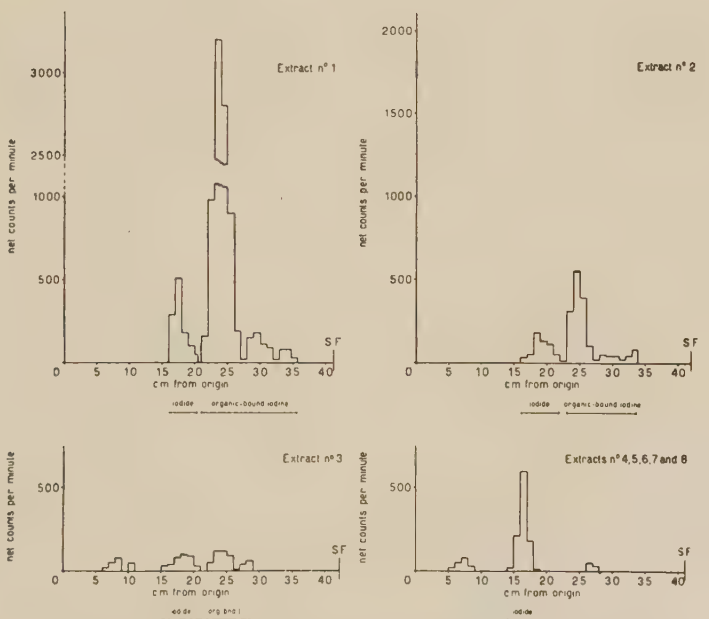

Fig. 3. I^{131} distribution on paper chromatograms of several n-butanol extracts obtained using a 2.5 : 1 relation between solvent and plasma volumes; pH 2—3. The first three extracts were run separately and the last five on the same paper.
The iodized compounds were separated by the same procedure and in the same sequence as in the case of the chromatograms shown in Fig. 1.

Table IV. *Percentage of serum I^{131} found in eight extracts and on the corresponding chromatograms, when the relation between n-butanol and sample volumes is 2.5: 1 and the pH 2—3.*

Per cent of sample I^{131}	Extract No. 1	Extract No. 2	Extract No. 3	Extract No. 4—8	Total
Extracted	57.3	23.4	8.2	9.2	98.1
Non-extracted					1.6
Present on the corresponding chromatogram as iodide	6.6	4.9	3.6	8.7	23.8
Present on the corresponding chromatogram as organic-bound iodine	49.7	17.5	4.8	0.2	72.2
Recovered (found on chromatograms + non-extracted)					97.6
Protein-precipitable (with Somogyi's reagent)					75.1

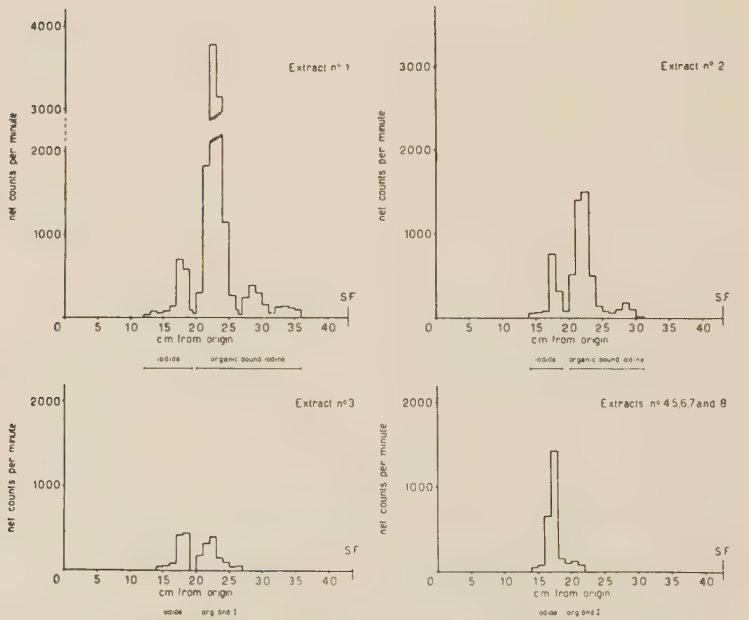

Fig. 4. I^{131} distribution on paper chromatograms of several n-butanol extracts obtained using a 2.5 : 1 relation between solvent and plasma volumes; pH 2—3. The first three extracts were run separately and the last five on the same paper.
The iodized compounds were separated by the same procedure and in the same sequence as in the case of the chromatograms shown in Fig. 1.

hyroxine peak. Moreover after the third xtraction only iodides are extracted, as can e concluded from Fig. 3, where the chro- hatogram of the 4th—8th extracts is shown. lmost 97 per cent of the initial radioactivity as extracted in eight times.

In the last experiment 2.0 ml of human erum, to which a mixture of I^{131} as iodide, hyroxine and tri-iodothyronine had been lded, was used.

The same extraction procedure as for the vo proceeding experiments was used, eight xtracts being obtained with 5.0 ml of n-butyl cohol each.

The results are summarized in Fig. 4 and able IV.

They are essentially the same as for the her experiments where the same proportion solvent to sample was used. Most of the dioactive organic compounds were extrac- d in the first two times; only 4.8 per cent the total activity of the sample appeared the third chromatogram in the thyroxine ot. On the fourth chromatogram no other mpounds but iodide were demonstrated. he total activity recovered on the chromato- ams as organic compounds correlates fairly ell with the protein precipitable.

DISCUSSION

When 4.0 ml n-butyl alcohol are used for 2.0 ml of plasma or serum, at least four extractions have to be carried out at pH 2—3 to free the sample of all its organic iodized compounds.

This extraction can be completed in three times, when 5.0 ml of solvent are used for the same amount of sample. If the third extraction is not carried out, only about 4 per cent of the iodized compounds is lost.

It seems that the activity remaining in the protein residue after three extractions is due only to iodides. This is sustained by the fact that the activity extracted as organic compounds correlates fairly well with that which is protein precipitable (with Somogyi reagent).

SUMMARY

1) If at least 5.0 ml n-butyl alcohol are used for 2.0 ml of plasma or serum at a pH 2—3, the extraction of the organic bound iodine is completed after three extractions.

2) The losses resulting if the third extract is not carried out are about 4 per cent of the total organic iodine compounds present in the sample.

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A SIMPLIFIED TECHNIQUE FOR REMOVAL OF COMPOUNDS INTERFERING IN CREATININE DETERMINATIONS

By S. SPANNER and R. J. BOSCOTT

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Most of the methods available for improving the specificity of the picric acid method in the determination of creatinine are relatively time consuming. (Taussky, 1954; Kostir & Sonka, 1952). Since the known interfering compounds are ketonic — aceto acetic, pyruvic, ascorbic and α-keto glutaric acids, and acetone — we have investigated the use of KBH_4 to reduce them prior to creatinine estimation. The method used is partly based on that of Bonsnes & Taussky (1945) for the determination of urinary creatinine.

Table I.

Solutions examined	Galvanometer readings	
	Method of Bonsnes & Tausskey (1945)	KBH_4 modified method
Creatine standard I.	0.39	0.39
I+lactic acid 200γ/ml ...	0.39	0.39
I+sodium pyruvate 200γ/ml	0.43	0.39
I+p-hydroxyphenylpyruvic acid 200y/ml.	0.49	0.39
I+ α-ketoglutaric acid 100γ/ml	0.54	0.38
I+ascorbic acid 500γ/ml..	0.45	0.39
I+d-glucose 500γ/ml	0.39	0.385
I+acetone 500γ/ml (14 hrs KBH_4 treatment)	0.635	0.39

METHOD

4 ml of standard creatinine solution with a without the added compounds were pipetted i a 10 ml graduated stoppered measuring cylind 25 mg of KBH_4 (May & Baker Ltd.) was add and the mixture allowed to stand 45 minutes S ficient 2 N HCl was then added to pH and t mixture stood for a further 30 minutes with occ sional thorough agitation to destroy the exc KBH_4. 2 N NaOH was then added to pH 6.5– and the volume adjusted to 6 ml with water. 3 of the final solutions were treated with alkali a picric acid and the color measured exactly as d scribed by Bosnes & Taussky (1945). Acetone w found to require longer exposure to KBH_4. T results are shown in Table I. The method has be applied to urines when 1.5 ml of urine was dilut to 100 ml and 4 ml of urine used in the procedu described instead of the 4 ml of creatinine standa

Preliminary work indicates that urines fr normals give almost identical values for creatini with and without interaction with KBH_4 where urines from patients with phenylketonuria give r duced values with KBH_4. We anticipate that urin from patients with deficient or greatly augment intake of ascorbic acid should give more accura values for creatinine by the new method. Urin containing acetone should be treated with KBH for 14 hrs. (overnight) otherwise interference m occur. Glucose and lactic acid do not interfe with the method. The method has not yet be applied to the determination of creatinine in blo

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DETECTION AND DETERMINATION OF PORPHOBILINOGEN IN URINE

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Porphobilinogen gives a red color when reated with Ehrlich's aldehyde reagent, p-imethylaminobenzaldehyde in acid solution. he colored compound is not extracted by hloroform from the reaction mixture either hile still acid or after addition of an equal olume of a saturated sodium acetate solu-on, and this forms the basis of the original achs (1931) and later Watson & Schwartz 1941) tests for the detection of porphobili-ogen in urine and its differentiation from robilinogen; the red color due to urobili-ogen is extractable by chloroform.

For the quantitative determination of por-hobilinogen in urine, Vahlquist (1939) used modified Ehrlich reagent containing a rger amount of p-dimethylaminobenzal-ehyde and omitted the addition of sodium cetate which causes precipitation of the dehyde at this concentration. Vahlquist alibrated the color afforded by porphobili-ogen-containing urines in terms of that ven by urobilinogen, using a Zeiss stufen-notometer, and defined one porphobilinogen nit as "Die Konzentration einer Lösung emeint, die beim Versetzen mit P. d. a [1] gleiche Teile) ein $E = \frac{1.09}{2} = 0.545$ zeigt. e hat dabei dieselbe Extinktion wie eine mg %ige Urobilinogen lösung im maxi-alen Extinktionsgebiet . . ."

There has been some uncertainty as to e interpretation of this definition (Westall, 1952; Cookson & Rimington, 1954; Watson, 1954) but since pure porphobilinogen has become available and the spectral absorption ($E^{\frac{1\ cm}{1\ \%}}$ at 552 mμ) of the red color it gives with Ehrlich's reagent under the specified conditions has been put on record, the less satisfactory 'porphobilinogen unit' may be discarded.

In the course of many studies involving the determination of porphobilinogen in human and animal urines we have observed that Vahlquist's method is far from satisfactory. He himself noted that certain unknown urinary constituents retarded the rate of color formation; moreover, the color is not stable but fades progressively so that he recommended an addition of 10 per cent to the color value actually measured. In some normal animal urines (*e. g.* rabbit), to which pure porphobilinogen has been added, we have found color formation to be completely suppressed until the addition of sodium acetate, unless comparatively large amounts of porphobilinogen are present; we have referred to such a color test as a 'pseudoporphobilinogen reaction' (Goldberg & Rimington, 1955). Urines from human patients with acute porphyria have sometimes exhibited the same phenomenon and when screening urines for the presence of porphobilinogen we have often been in doubt whether such weak reaction was due to porphobilinogen or to some other substance. Wherever possible, we confirm the urinary color test by paper chromatography since urines may contain a vast number of Ehrlich positive substances

[1] P. d. a. = Ehrlich's reagent.

and in some cases the colored complexes are not extractable by chloroform (*e. g.* tryptophan) and may then lead to erroneous conclusions. We cannot stress too strongly the importance of this chromatographic verification.

Formijne and Poulie (1954) endeavoured to eliminate the errors in the Vahlquist method by diluting the urine sample always to a S. G. of 1·0005 before measurement and then applying a standard correction of + 9 per cent. They also use a reagent containing ethanol which gives a slower, but, in their experience, a more regular rate of color development.

In this paper we present a study of the reaction between p-dimethylaminobenzaldehyde and porphobilinogen in aqueous solution or in urine, and propose, for the determination of porphobilinogen in pure solution or simple extracts, the use of a modified Ehrlich's reagent which contains acetic acid in addition to hydrochloric acid and affords a much greater intensity of color. Experiments are recorded upon substances inhibiting the reaction and the nature of the inhibitors present in rabbit urine. A simple technique is described for the quantitative separation of porphobilinogen from the inhibiting substances present in urine. The chemistry of the Ehrlich reaction is discussed in the light of recent work by Treibs & Derra-Scherer (1955).

EXPERIMENTAL

MATERIALS AND METHODS

Porphobilinogen: isolated from acute porphyria urine and used in the form of the freshly recrystallized hydrochloride (Cookson & Rimington, 1954).

Ehrlich's reagent I (as used by Watson & Schwartz, 1941) — 0.7 g p-dimethylaminobenzaldehyde dissolved in 150 ml conc. HCl + 100 ml H_2O.

Ehrlich's reagent II (as used by Vahlquist, 1939): 5 g p-dimethylaminobenzaldehyde dissolved in 125 ml conc. HCl + 125 ml H_2O.

Ehrlich's reagent III (as recommended in this paper): 15 g p-dimethylaminobenzaldehyde dissolved in 65.6 ml conc. HCl + 194.4 ml glacial acetic acid.

Glucuronides and glucuronic acid: kindly supplied by Professor R. T. Williams, St. Mary's Hospital, London.

Photoelectric spectrophotometer: Unicam S. P. 500.

Measurements were made at the maximum found for the porphobilinogen-aldehyde reaction product (ca. 552 mμ).

RESULTS

Relation between color intensity and porphobilinogen concentration in water and in urine

Pure solutions.

Ehrlich's reagent II (1 vol.) was added to aqueous porphobilinogen solutions (1 vol.) and in each mixture readings of density at 552 mμ taken in rapid succession. A plot of the highest reading reached against porphobilinogen concentration affords a straight line in accordance with Beer's law. (Fig. 1.)

Solutions in urine.

From a human urine containing added porphobilinogen, serial dilutions with water were prepared and each treated with Ehrlich's reagent II and measured as above. The plot (Fig. 2) shows that Beer's law is not obeyed; proportionately less color is devel-

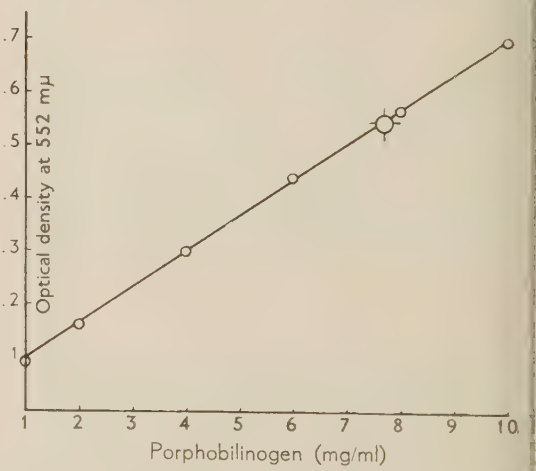
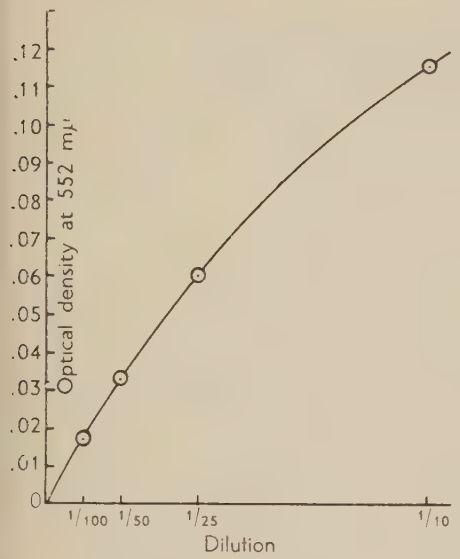


Fig. 1. Beer's law relationship between concentration of porphobilinogen in water and optical density at 552 mμ of its reaction product with Ehrlich's reagent II.



g. 2. Relationship between concentration of porbilinogen in normal human urine and optical denty at 552 mμ of its reaction product with Ehrlich's reagent II. Beer's law is not obeyed.

ed in the more concentrated solutions. If, wever, the logarithm of the dilution is otted against the logarithm of the optical nsity, as in Fig. 3, a straight line is obined. Examining different urines in this ay one gets a series of such lines but the pe is not constant. Precipitation of the rphobilinogen from the urine by mercuric etate and recovery by means of H_2S does t remove the interfering substance(s), the g. D: log. dilution line remains linear hough altered in slope (Fig. 3).

Acute porphyria urines, containing unown quantities of porphobilinogen, behave the same way but since the amount of rphobilinogen present is usually very high, en permitting a 50 or 100 fold dilution fore measurement, the departure from er's law is less noticeable at the higher ution (see Fig. 7). A mathematical pression could almost certainly be derived hich would give the true porphobilinogen ntent of any urine from observations made selected dilutions with and without the

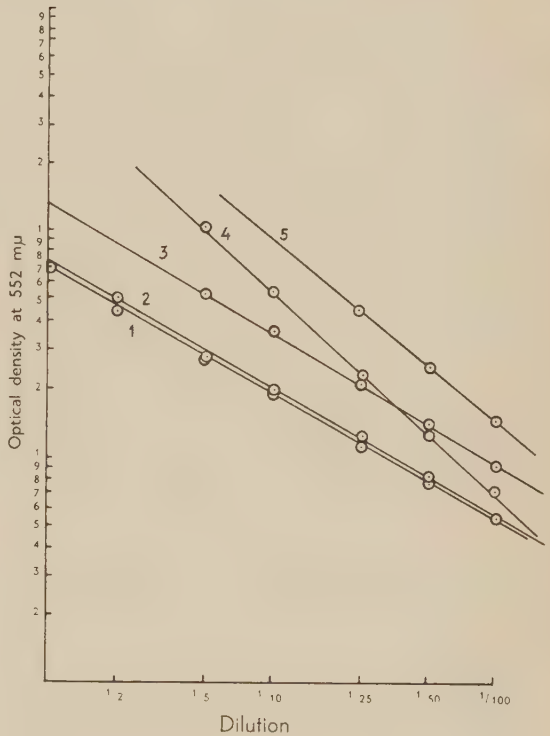


Fig. 3. Logarithmic plot of optical density developed at 552 mμ with Ehrlich's reagent II and porphobilinogen in human urine at various dilutions with water.

1. 40 μg/ml added to normal urine, SG 1.0145.
2. Acute porphyria urine.
3. 100 μg/ml added to normal urine, SG 1.0175.
4. Acute porphyria urine pptd by Hg acetate; ppt decomposed.
5. 200 μg/ml added to urine, SG 1.0140, containing much indican.

addition of a small known quantity of porphobilinogen. This possibility has not been further pursued, in view of the successful use of ion-exchange resins for elimination of the inhibitory substances (see later).

Enhancement of color by acetic acid

It was noticed that with aqueous solutions of porphobilinogen the presence of acetic acid increased color intensity in the reaction, other things being equal. The reason for this is obscure; it is thought to be a 'solvent effect'

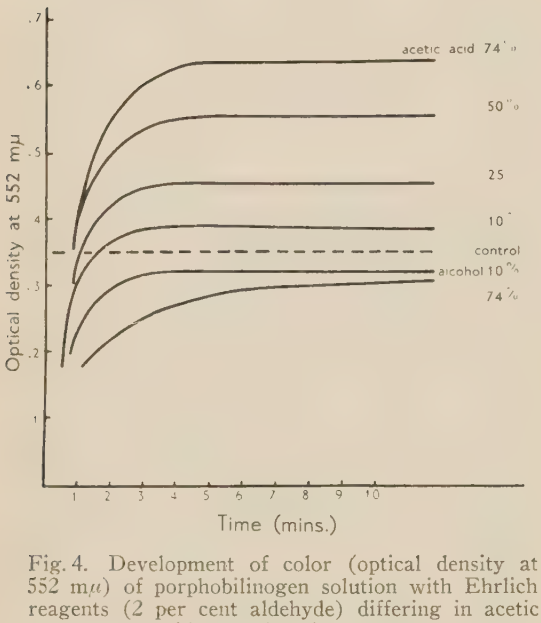


Fig. 4. Development of color (optical density at 552 mμ) of porphobilinogen solution with Ehrlich reagents (2 per cent aldehyde) differing in acetic acid or ethanol content.

since it is certainly not due merely to an increase in acidity. In all subsequent experiments a reagent has been used containing 78 per cent (v/v) of acetic acid. Ethanol has the opposite effect of depressing color formation (Fig. 4).

Effect of changing the concentration of p-dimethylaminobenzaldehyde

Ehrlich's reagent II contains 2 per cent of the aldehyde. Reagents were prepared containing 78 per cent (v/v) acetic acid, 3 *M*-HCl and amounts of aldehyde ranging from 0.28 to 6 per cent and the color developed by these when added to the same amount of porphobilinogen (chosen to give $D_{555} \triangleq 0.6$, the maximum extinction being at this wavelength in solutions containing acetic acid) was examined as regards both speed of development and final intensity. The results are shown in Fig. 5 and indicate that speed of color development increased with aldehyde concentration. The final optical density was independent of aldehyde concentration.

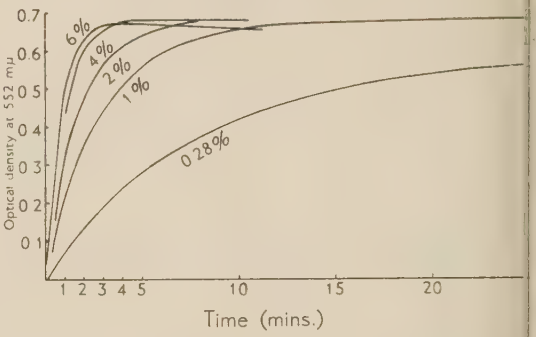
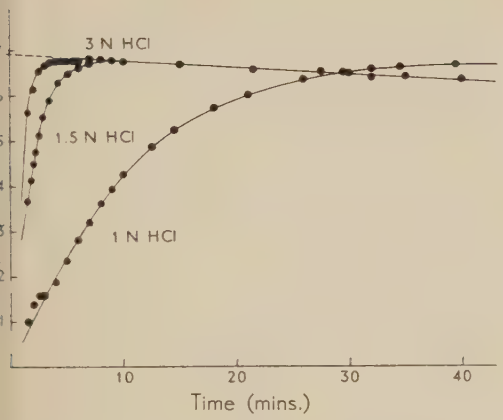


Fig. 5. Rate of color development and final intensity (optical density at 555 mμ) of a porphobilinogen solution with Ehrlich's reagent 3 *M* in HCl and containing 78 per cent (v/v) of acetic acid together with 0.28, 1, 2, 4 or 6 per cent aldehyde.

Effect of changing the concentration of HCl

Reagents were made up containing 8 per cent p-dimethylaminobenzaldehyde (in order to obtain rapid color development), 78 per cent (v/v) acetic acid, water and HCl to give final HCl molarities in the reaction mixture of from 0.5 to 1.5 (*i. e.* reagents 1 to 3 *M* in HCl). The velocity of color development with the same quantity of porphobilinogen was then measured as before. The results shown in Fig. 6 indicate that although the final density remained constant, the velocity of color development increased with increasing HCl concentration. In every case, fading occurred after the maximum was attained and the rate of this fading appeared to be linear and independent of acid concentration. Porphobilinogen is known to be converted slowly into uroporphyrins in acid solution and it seemed possible that some such change as this might be responsible for the fading. That it is due rather to some change in the color complex itself was proved as follows. A porphobilinogen solution in 1.5 *N*-HCl was divided into two portions, A and B. Ehrlich reagent made with 1.5 *M*-HCl was added to A immediately and the rates of color development and fading were recorded. After 100 mins., by which time the color of



ig. 6. Rate of color development (optical density 555 mμ) and fading of a porphobilinogen solu-on with Ehrlich's reagents each containing 8 per nt of aldehyde and 78 per cent (v/v) of acetic acid but either *1 M*, 1.5 *M* or 3 *M* with respect to HCl.

had faded 25 per cent, the reagent was lded to B. The same color intensity and ite of fading were recorded as in A. There ould not thus have been any significant de-ruction of the porphobilinogen whilst xposed to HCl alone. We have observed at fluorescence due to porphyrin develops ore readily in acid solutions of porphobili-ogen when Ehrlich's reagent is present but e reaction mechanism by which porphyrin thus formed is almost certainly different om that obtaining in simple acid solution see discussion).

roposed modification of Ehrlich reagent for porphobilinogen

In chosing the most suitable reagent which ill react rapidly with porphobilinogen and ford an intense color, one has to allow for e time taken to insert the cells in a spectro-notometer and carry out the necessary anipulations to obtain a reading. By selec-ng a reagent which is 3 *M* in HCl, approxi-ately 5 minutes elapse between mixing and evelopment of maximum optical density. Fading during this time is relatively very small, but can be allowed for, if desired, by continuing the readings so that the slope of the linear fading line is obtained and then extrapolating to zero time (cf. Fig. 6). We find a reagent containing 6 per cent (w/v) p-dimethylaminobenzaldehyde, 78 per cent (v/v) acetic acid and HCl to a molarity of 3 to be most generally satisfactory (Ehrlich reagent III); there is, however,a formation of small bubbles on mixing test solution and reagent; they may be removed by tapping. Using this reagent and measuring the optical density at 555 mμ, porphobilinogen gives $E\,{}^{1\,0/0}_{1\ cm} = 1 \cdot 136 \times 10^3$. Since for the determination the porphobilinogen solution is diluted with an equal volume of reagent, this corresponds to apparent ε mol = 53,640 for the color complex itself.

Order of reaction and nature of the colored complex.

From the data expressed by Fig. 6 deductions can be drawn concerning the order of reaction with respect to porphobilinogen, the aldehyde being present in very large excess and the maximum attained rapidly in 1.5 *M*-HCl(final concentration) being assumed to measure the total concentration of porphobilinogen. The slow reaction in 0.5 *M*-HCl shows a first order kinetics (Table I) hence the colored complex appears to be a monopyrrolic derivative (see further below). Attempts to discover the order of reaction with regard to p-dimethylaminobenzaldehyde encounter difficulty on account of the fact that the colored complex is decomposed, soon after its formation, into other products, the colored solutions acquiring a purplish tint and exhibiting a spectral absorption very different from that of the primary pink reaction product. A plot of the color development/time curve has the configuration typical of consecutive reactions, the red colored product being the intermediate.

Table I. *Order of reaction between porphobilinogen and Ehrlich reagent containing N-HCl (i.e. final concentration in mixture 0.5 N) with aldehyde in great excess (see Fig. 6).*

t	a	x	a—x	$\frac{1}{t} \log \frac{a}{a-x}$
0	0.690			
5		0.250	0.440	0.0391
10		0.426	0.264	0.0417
15		0.533	0.157	0.0429
20		0.593	0.097	0.0426
25		0.631	0.059	0.0427
30		0.652	0.038	0.0420
35		0.663	0.027	0.0402

Modification of the color reaction by various substances

As stated above, porphobilinogen added to normal rabbit urine fails to give any color with Ehrlich's reagent but does so after the further addition of saturated sodium acetate solution ('pseudoporphobilinogen reaction', Goldberg & Rimington, 1955). For convenience, the color produced by the addition of reagent alone will henceforth be referred to as the 'primary color' and that appearing after addition of sodium acetate as the 'secondary color'.

Various substances were tested for possible influence on the color reaction between pure aqueous porphobilinogen and Ehrlich's reagent I and the results are summarised in Table II. It will be seen that the primary color is inhibited by thiols and by ergothioneine disulphide. Cystine, however, is non-inhibitory as is S-methylcysteine sulphoxide. Secondary color develops with all but cysteine. Those substances inhibiting the primary color will also bring about rapid fading if added after the color has been fully developed with Ehrlich's reagent.

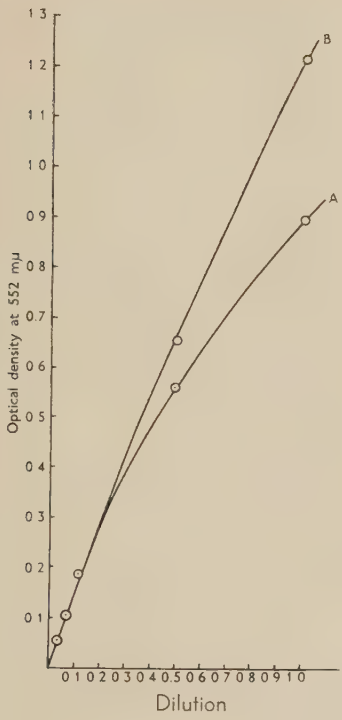
Suppressed primary color may be caused to develop by the addition of oxidising agents such as $KClO_3$, $K_2Cr_2O_7$,*etc.*, or of mercuric acetate which can be regarded either as an oxidant or as a complexing agent. Thallous salts behave similarly.

Table II. *Effect of various substances upon the color reaction between aqueous porphobilinogen and Ehrlich reagent I.*

Substance added	Color on addition of reagent (primary color)	Color on further addition of Na acetate (secondary color)
Cystine	+ +	
Cysteine	(+) fading	—
S-Methylcysteine sulphoxide	+	
Ergothioneine	—	+ +
Ergothioneine disulphide	—	+ +
2-thiol-histidine	—	+ +
Thiourea	—	+ +
Thiouracil	+ +	
1-methyl-2-thiolimidazole	—	+
d, 1-5-vinyl-2-thio-oxazolidone	+ +	
Urea	+ +	
Uric acid	+ +	
Rabbit urine	—	+ +
Human urine	+ +	

The inhibitor of rabbit urine

The inhibitor present in rabbit urine behaves, on the whole, as do the known thiol inhibitors but its nature must be different since it is not precipitated by thallous salts. Concentrates of the urinary inhibitor, prepared by paper chromatography, fail to give tests for -SH but do always give a naphtho-resorcinol test for glucuronides. A number of pure glucuronides, glucuronic acid and glucurone were tested but found to have no inhibitory action. By subjecting rabbit urine containing porphobilinogen to paper chromatography solvent (butanol 63; acetic acid 11; water 26), porphobilinogen runs to its usual position where it can be detected by spraying



'ig. 7. Behavior of porphobilinogen; *A* added to ormal human urine; *B* in the eluate after chromatography of the mixture on Dowex 2.

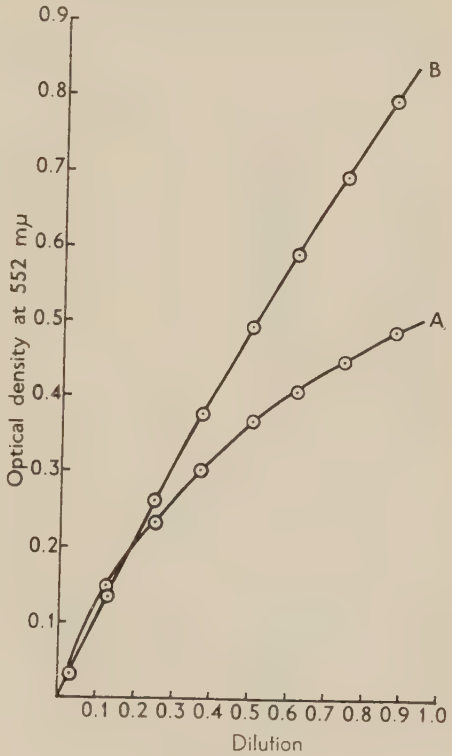


Fig. 8. Behavior of porphobilinogen; *A* in acute porphyria urine; *B* in the eluate after chromatography of the urine on Dowex 2.

vith Ehrlich's reagent (Cookson & Rimington, 1954); the inhibitor remains on the starting line and may be detected there as a olorless spot on a red ground by spraying ne paper lightly with porphobilinogen and nen with Ehrlich's reagent. Exactly the ame behavior is observed whether pure porhobilinogen is added to normal rabbit urine r whether it be excreted by an animal dosed vith allyl-isopropyl-acetamide. Partial sepaation of porphobilinogen and inhibitor is lso accomplished using a column of Al_2O_3. hould there be any combination between orphobilinogen and the unknown inhibitor must be of a very weak character.

Separation of porphobilinogen and urinary inhibitor by ion-exchange. Quantitative determination of porphobilinogen in urine.

Porphobilinogen is known to be retained by suitable ion-exchange resins (Westall, 1952, Granick & Vanden Schrieck, 1955) and their use offered the possibility of separating porphobilinogen quantitatively from other substances in urine which interfere with its quantitative determination by the Ehrlich reagent. As already mentioned, Beer's law is not obeyed in the presence of urine (see Fig. 2).

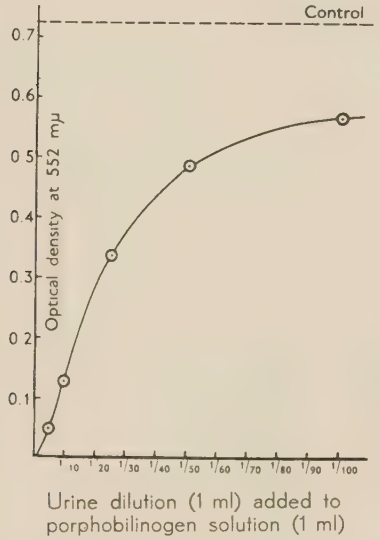


Fig. 9. Effect of addition of dilutions (1 ml) of rabbit urine upon color development by porphobilinogen (1 ml) and Ehrlichs's reagent II (2 ml). The control line represents the optical density developed with the reagent by this same amount of porphobilinogen dissolved in water. Even on addition of a 1/100 dilution of rabbit urine there is an inhibition of color development of over 20 per cent, with a 1/10 dilution, 82 per cent.

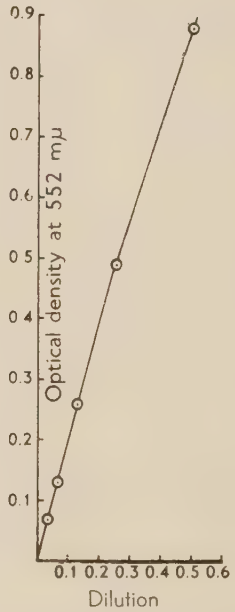
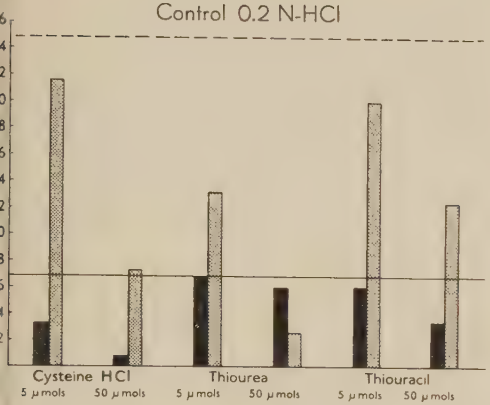


Fig. 10. Behavior of the eluate after chromatography of a rabbit urine (0.75 ml) + porphobilinogen solution (0.25 ml) on Dowex 2.

We have found that on passing a porphobilinogen-containing urine (2 ml) through a 6 cm × 1 cm colum of 'Dowex' 2 (50—100 mesh; 8 per cent cross linkage) previously treated with 2 *N*-NaOH and then washed until neutral, the porphobilinogen is quantitatively retained whilst inhibitory and interfering substances are eliminated in the filtrate and water washings (25 ml). 0.5 *M*-ammonium acetate displaced the porphobilinogen from the column (a volume of 50 ml was usually employed) and Beer's law is obeyed in these eluates over a considerable range. Recoveries of porphobilinogen added to normal human or rabbit urines have varied between 91 per cent and 100 per cent. Figs. 7—10 illustrate some of the experiments performed.

Formation of porphyrins from porphobilinogen

In a recent paper, Watson (1954) has suggested that two forms of porphobilinogen or two porphobilinogens may occur in urines, the one yielding porphyrin when the urine is heated within a pH range of 2 to 6, the other requiring acidification to pH 0.7 to 0.8 and heating to produce any porphyrin. He suggests that in urine of the latter type porphobilinogen may be complexed with uric acid. No correlation is reported between acidity required for porphyrin formation and behavior with Ehrlich's reagent, although Watson has apparently observed the two types of color reaction noted above. As stated in Table II, we find addition of uric acid to aqueous porphobilinogen to have no effect upon the color test with Ehrlich's reagent.



ig. 11. Yields of uroporphyrin (from optical den-ty measurements at 403 mμ in N-HCl) from por-obilinogen (1μMol) + various inhibitors (5 and) μMols) heated for 20 mins in either acetate uffer pH 4.2 (black columns) or 0.2 N-HCl giving nal pH of 0.7 (dotted columns). The yields of oporphyrin in these two systems, but in absence any inhibitor, are indicated by the full and dotted lines respectively.

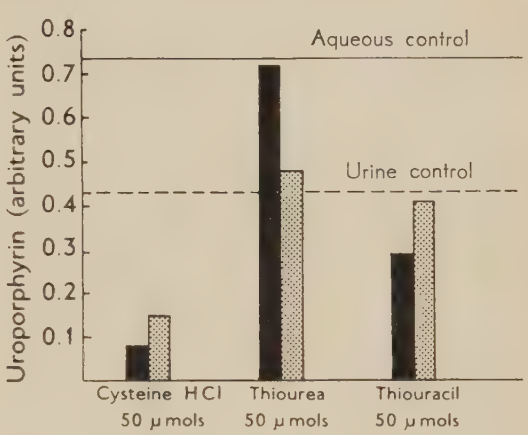


Fig. 12. Comparison of yields of uroporphyrin (from optical density measurements at 403 mμ in N-HCl) from porphobilinogen (1 μMol) heated for 20 mins with acetate buffer pH 4.2 and various inhibitors (50 μMols) in presence of either *water* or *urine*. The yields in absence of any inhibitor are indicated by the full and dotted lines for the water system and urine system respectively. Black colums aqueous medium; dotted columns, in presence of urine.

To clarify this situation, certain experi-ents have been performed as follows, the ffect of heating porphobilinogen in 0.2 -HCl or in acetate buffer pH 4.2 in the resence of various inhibitors being observed a) in aqueous solution and (b) in normal rine instead of water.

Reagents used

	Porphobilinogen	2.5
i	Cysteine hydrochloride	78.5
ii	» »	7.85
i	Thiourea	38.0
ii	»	3.8
i	Thiouracil	64.0
ii	»	6.4
i	Water	
ii	»	

In the first set of experiments (a) mixtures ere made of A (0.1 ml) + Bi or Bii . . . *etc.* 0.1 ml) + 2 ml of acetate buffer pH 4.2 2 M). A similar set of tubes contained 2 ml 0.2 N-HCl in place of acetate buffer; this ave a final pH of approximately 0.7. All tubes thus contained 1 μM of porphobilinogen and either 5 or 50 μM of an inhibitor (water in the two controls). They were heated in a boiling water bath for 20 minutes, cooled and 2.2 ml of 2 N-HCl added. Dilutions were then made with N-HCl as required and the optical density measured at 403 mμ (Soret maximum of uroporphyrin in this solvent). The results are shown in Fig. 11.

In the second series of experiments (b), 1 μM of porphobilinogen was used as before and 50 μM of either cysteine HCl, thiourea or thiouracil; the quantity of acetate buffer pH 4.2 or 0.4 N-HCl was 1 ml and the total volume (2.2 ml) was made up by addition of either water (1 ml) or fresh urine (1 ml). Heating and determination of uroporphyrin was carried out as before and afforded the results plotted in Figs. 12 and 13.

From Figs. 11—13 several conclusions may be drawn, as follows; (1) In aqueous solution, the yield of uroporphyrin from porphobilinogen is much higher at pH 0.7 than

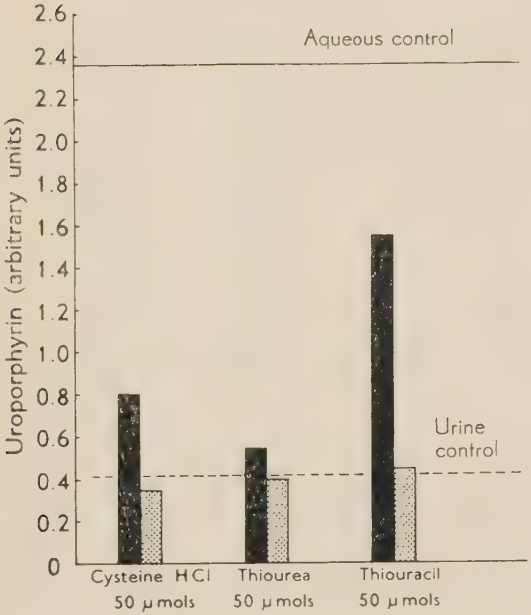


Fig. 13. Data as for Fig. 12 but 0.2 *N*-HCl (pH app. 0.7) used in place of acetate buffer. Black columns, aqueous medium; dotted columns, in presence of urine.

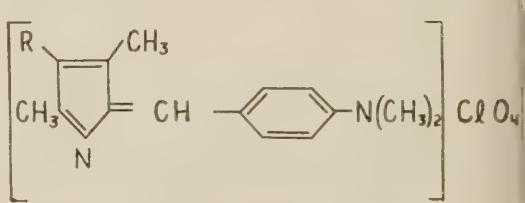
in buffer at pH 4.2 but in the presence of urine (ca. 1/2 of the total vol.) this difference is no longer apparent, in fact slightly more uroporphyrin is formed at pH 4.2 than at 0.7. These findings are in agreement with unrelated observations of Westall (1952), Cookson & Rimington (1954) and Grieg, Askevold & Sveinsson (1950). (2) The substances used cause varying degrees of inhibition, usually more at pH 4.2 than at pH 0.7 but *vice versa* in the case of thiourea. There is no constant relationship from one substance to another in the extent of inhibition induced at these two pH's. (3) The presence of urine profoundly affects the degree of inhibition in each case. Thus thiouracil exerts practically no inhibition *in urine* at either pH 4.2 or 0.7 but inhibits greatly at both pH's in a water medium. Thiourea is without inhibition except at pH 0.7 *in water*. Cysteine HCl (50 μM) shows a more complicated behavior. Inhibitions of between 66 per cent and 90 per cent are seen at either pH in water and at pH 4.2 in urine, but at pH 0.7 *in urine* the yield of porphyrin is nearly the same as in the absence of cysteine.

The interpretation of these results is clearly difficult; one inference might be that the inhibitors form complexes with some component of the chromogenic system, the dissociation of the complex depending upon pH and varying for each inhibitor. Even normal urine profoundly affects the system, greatly lowering the yield of uroporphyrin (as compared with a watery solution) at pH 4.2 and still more markedly at pH 0.7. These lowered yields may be due to non-oxidation of the porphyrinogen first formed, a point not further investigated as we are primarily concerned with the results of analyses and tests as usually carried out. One point is clear. Our results could explain in simple terms Watson's (1954) experience with certain porphobilinogen-containing urines. Postulation of two porphobilinogens is unnecessary. Thus, in presence of normal urine to which porphobilinogen has been added and an appropriate quantity of cysteine, heating at pH 4.2 will give practically no porphyrin, whilst at pH 0.7 the same mixture will yield nearly ten times as much. We suggest that his effects were caused in an analogous manner.

DISCUSSION

Chemical mechanism of the Ehrlich reaction

According to Fischer, pyrroles and p-dimethylaminobenzaldehyde react at elevated temperature (boiling ethanolic solution) to give dipyrrylphenylmethane derivatives, but at room temperature a mole-to-mole reaction ensues. Thus Fischer & Nenitzescu (1925) isolated and characterized the perchlorates I and II.

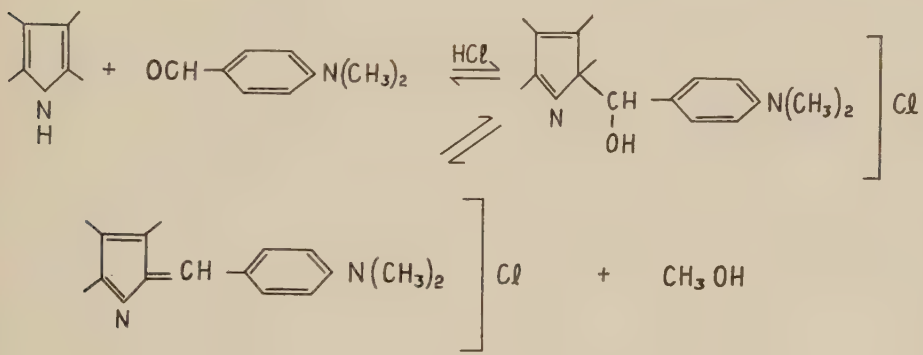


I $R = COOC_2H_5$

I $R = CH_2CH(COOH)_2$

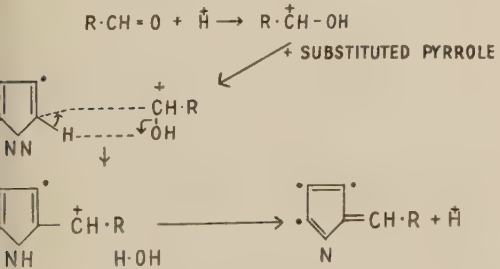
From II the yellow color-base was isolated as disodium salt. Its solution in CO_2-free water was yellow but became red on addition of small amounts of acid (even CO_2) ; excess of HCl led to decolorization, atributed to salt formation on the dimethylamino group.

Treibs & Derra-Scherer (1955) and Treibs & Herrmann (1955) have recently reinvestigated this whole subject. They show that, contrary to former belief, not only pyrroles with a free α-position but also tetraalkyl-pyrroles such as 2, 3, 4, 5 tetramethylpyrrole react with Ehrlich's reagent with the elimination of one alkyl group, probably as follows (/ denote CH_3 groups) :



A similar mechanism can be visualized for the pyrroles possessing a free α-position (H in place of CH_3). The equilibrium conditions are much affected by factors such as temperature and nature and concentration of the acid present.

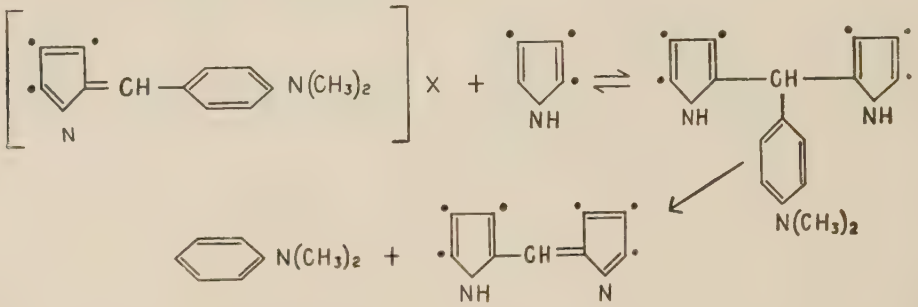
No definite evidence could be obtained for the presence of the carbinol intermediate. We consider that an alternative reaction mechanism may be considered, namely



According to this scheme, the carbinol has no real existence under the strongly acid conditions; it might be formed on alkalinization of the final product (cf. Goldacre & Phillips, 1949).

Treibs & Herrmann (1955) were able to demonstrate that further reactions take place after the formation of the pyrrole-Ehrlich aldehyde condensation compound. Further addition of a pyrrole unit leads to formation of a colorless dipyrrylphenylmethane and this in turn may lose dimethylaniline. The former reaction, but not the latter, is reversible. Formation of the Ehrlich dye was favored by alkyl substituents and one free α-position in the pyrrole nucleus and by excess aldehyde reagent; electro-negative substituents and pyrrole excess favored formation of the dipyrrylphenylmethane. The ease of elimination of dimethylaniline again depends upon the character of the pyrrole

unit but in nearly all cases the brown pyrromethene is formed on heating, the spectral absorption shifting to shorter wavelengths (see following formula).



Many of our observations with porphobilinogen become readily intelligible in the light of these studies of the pyrrole-Ehrlich aldehyde reaction. Thus, the spontaneous fading of the color which takes place after formation of the primary condensation product is probably due to formation of a dipyrrylphenylmethane derivative and eventually a dipyrrylmethene. The thiols which we have investigated and the unknown inhibitor in rabbit urine probably cause decolorization, or inhibit color formation by displacing the equilibrium normally attained. Thus one possibility would be competition in the strongly acid solution for the free α-position of the pyrrole nucleus.

ADDENDUM

Since this paper was written, a communication by Mauzerall & Granick (1956) has appeared in which methods are proposed for the determination of δ-aminolaevulic acid and porphobilinogen in urine. The latter is separated by Dowex 2 and an Ehrlich reagent is described containing perchloric and acetic acids. A suitable time is allowed for color development. The reaction is discussed briefly in the light of Treibs & Herrmann's work (1955).

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Scandinav. J. Clin. & Lab. Investigation 263—264, 8, 1956.

HYPO-URICEMIA DURING PREGNANCY

(Received for publication May 30, 1956)

It is known that the blood uric acid increases during pregnancy. Thus, Caldwell & Lyle (1921), in 150 normal pregnant women, found an increase in the blood uric acid from 15.3 μg/ml (6th month) to 21.3 μg/ml (8th month). Harding, Allin & van Wyck (1924) observed in 67 normal cases during pregnancy an increase from 26.1 μg/ml (2nd—6th month) to 30.8 μg/ml (7th—8th month and 34.8 μg/ml at labor. These authors used the method of Benedict (1922).

Apparently, none of the authors mentioned have compared pregnant and non-pregnant women. Such an investigation was therefore carried out. The plasma uric acid was measured by the method described by Prætorius & Poulsen (1953).

The experimental material consists of 359 uric acid determinations on 280 women. The ages of the pregnant women range from 17 to 46 years, and those of the non-pregnant from 18 to 52 years. No significant difference between the average ages of the two groups was demonstrated. The blood samples from the pregnant women were drawn in the morning when they presented themselves for prenatal control. None of the pregnant women had been treated for hyperemesis and they were all found to be perfectly normal. All the women in the non-pregnant group were patients with minor gynecological diseases. The blood samples from the control group were drawn in the morning on the day of admission.

The average uric acid concentrations found in plasma during the various months of pregnancy are compared with those of the non-pregnant in Table I. It appears that the average plasma uric acid is nearly constant during the first 7 months of pregnancy and at a lower level than usual for non-pregnant women (Group VII). It should be mentioned that Gjørup, Poulsen & Prætorius (1955) in their series of normal non-pregnant women have found an average value for serum uric acid (20—50 years) of 36.6 μg/ml. The difference between Groups I, II, III and Group VII is significant. In the 8th to 9th month of pregnancy the plasma uric acid rises significantly to the level of the non-pregnant women. In accordance with previous studies, Crawford (1939), an unquestionable increase is demonstrated during uncomplicated labour.

The results show that there is a moderate hypo-uricemia during the greater part of normal pregnancy.

ACKNOWLEDGMENT

The author wishes to thank Associate Professor E. Prætorius, M. D., Department of Pharmacology, University of Copenhagen, and Løvens Kemiske Fabrik, Copenhagen, for their generosity in supplying uricase for the determinations.

Table I. *Plasma uric acid in various months of normal pregnancy and in non-pregnant women.*

Group	Month of pregnancy	Cases	Plasma uric acid μg/ml	S. D.
I	2—3	93	29.5	5.5
II	4—5	43	29.4	5.3
III	6—7	15	30.5	5.0
IV	8—9	90	36.0	7.5
V	Labor	61	50.0	11.5
VI	Puerperium	12	36.6	6.8
VII	Non-pregn.	45	37.0	9.5

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Department of Biochemistry, and
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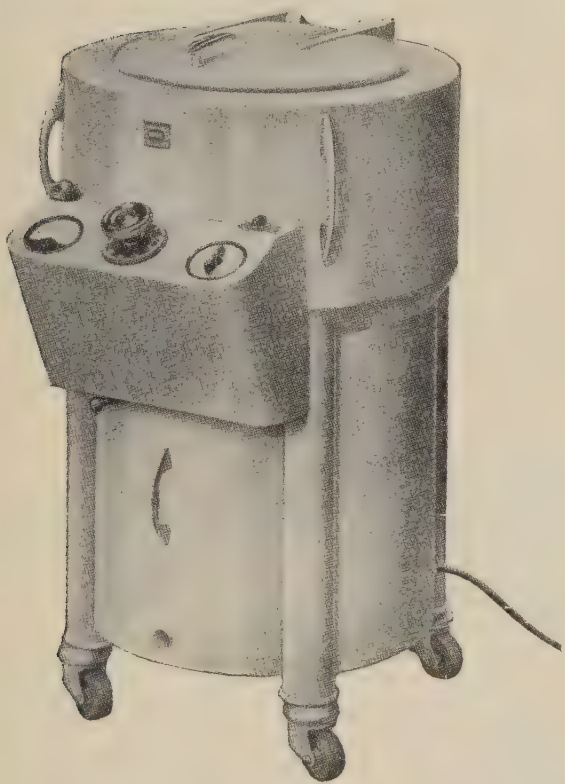

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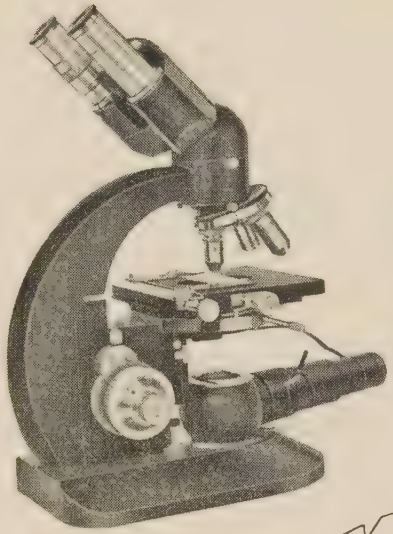

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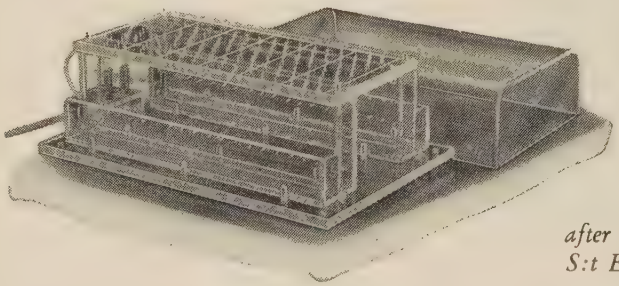

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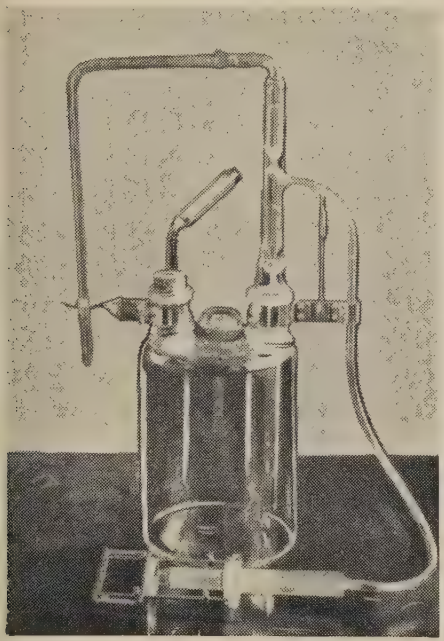

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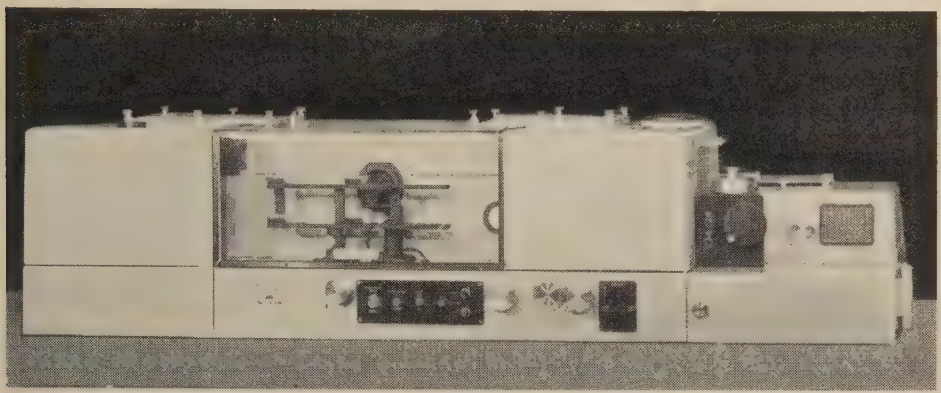

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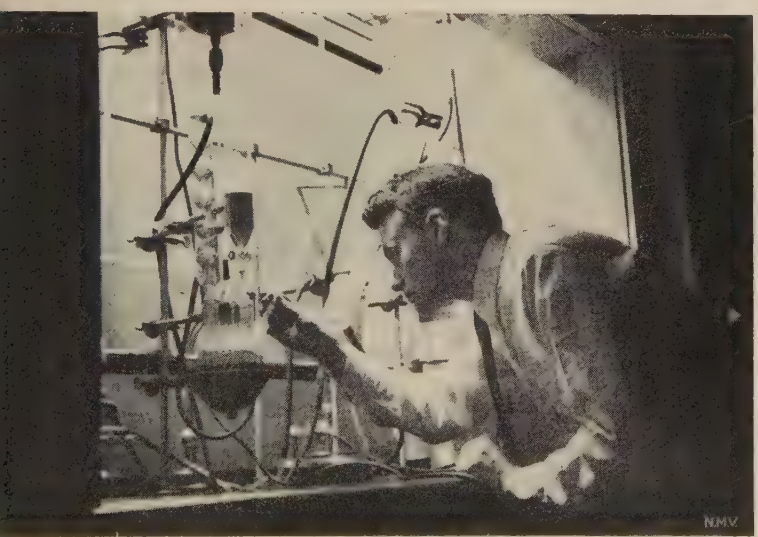

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